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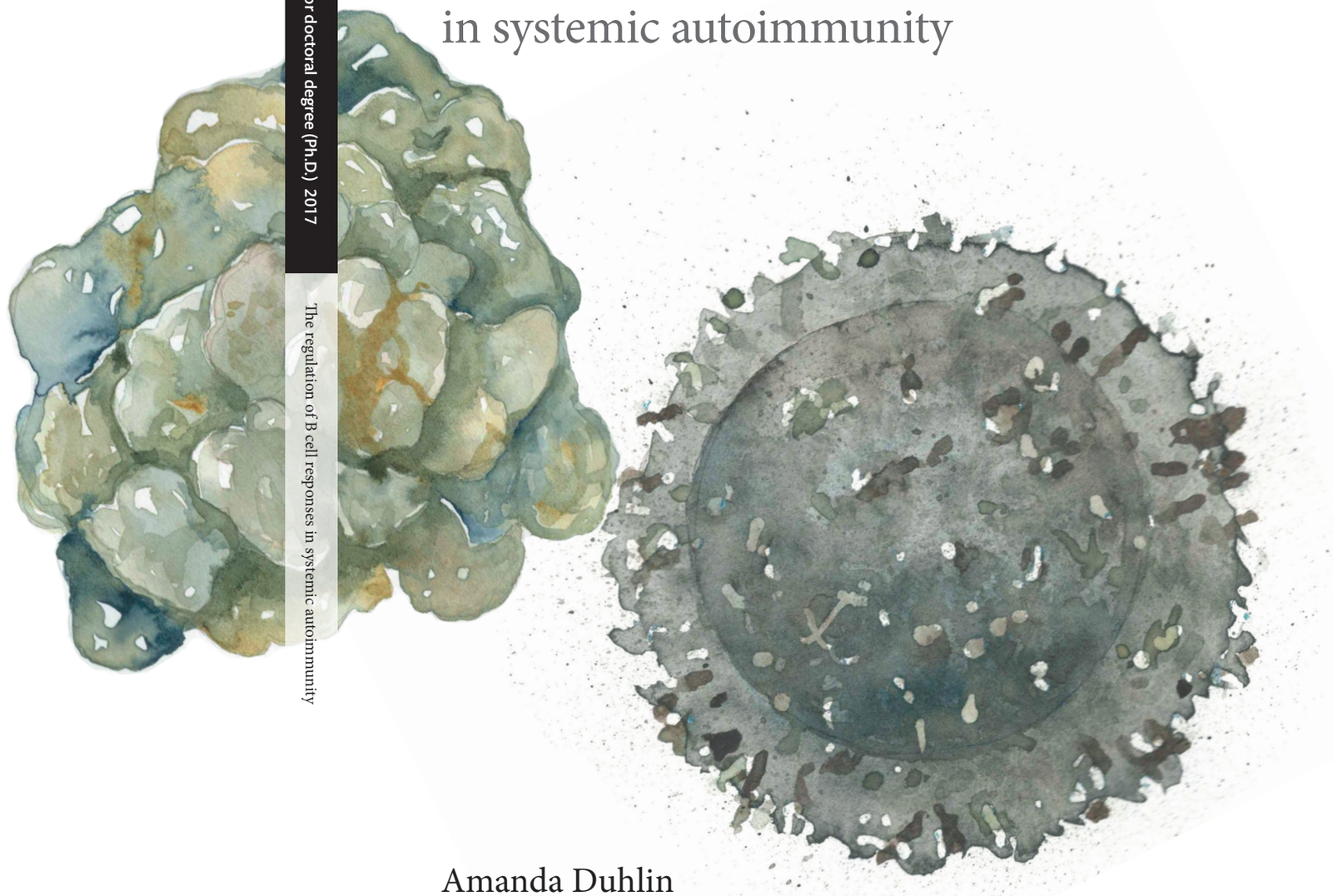

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# The regulation of B cell responses in systemic autoimmunity

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# ABSTRACT

Our immune system is a complex network made up of physical barriers and specialized proteins, cells and organs that all work together to prevent pathogens from causing disease in the body. Once the immune system has successfully mounted an immune response upon intrusion of a pathogen it will mount an immediate and stronger response against any subsequent exposure to it. This is known as immunological memory and is crucial for generating long-lasting protective immunity. The immune system has also developed to maintain homeostasis and be tolerant to the presence of the body's own structures, or so called self-antigens. A loss of this tolerance can lead to the immune system attacking the body itself, causing autoimmune disease. The pathogenesis of autoimmune disease involves both genetic and environmental factors. B cells and autoantibodies are major contributors to several autoimmune diseases such as systemic lupus erythematosus (SLE).

The aim of this thesis was to investigate the regulation of B cell responses in systemic autoimmune disease. This was studied in mouse models of autoimmunity and atherosclerosis and in paper III also in SLE patient samples.

**Paper I** was prompted by a study where transfer of spleen B cells from old atherosclerosis-prone apolipoprotein E-deficient (*ApoE*$^{-/-}$) mice to young *ApoE*$^{-/-}$ mice conferred protection against plaque development. We characterized the B cell response in the spleen of atherosclerotic *ApoE*$^{-/-}$ mice and found an ongoing B cell response in the form of germinal center B cells and plasma cells. Repeated injections of apoptotic cells, carrying the same oxidation-specific epitopes as oxidized LDL, into young *ApoE*$^{-/-}$ mice led to the same activated phenotype, lowered cholesterol levels and protected against plaque development. In **paper II** the memory response to apoptotic cell-derived self-antigens was characterized. Upon primary immunization of apoptotic cells a transient autoantibody response against the self-antigens DNA and phosphorylcholine was induced and when the primary response had waned, a single boost injection of apoptotic cells led to a rapid induction of the same autoantibodies. In a second recall response to apoptotic cells, mice presented with signs of autoimmune pathology such as IgG-deposition in the kidneys, positive anti-nuclear staining of antibodies from sera and altered architecture of the glomeruli indicating kidney damage. In **paper III** a role for the scavenger receptor CD36 on B cells was investigated in the context of apoptotic cell-derived self-antigens. CD36 inhibited B cell activation in the response to apoptotic cells and associated with known negative regulators of autoimmunity; the tyrosine kinase Lyn and FcγRIIB. Upon break of tolerance to the administered apoptotic cells and the activation of autoreactive B cells, the level of CD36-expressing marginal zone B cells was dramatically decreased and the same population of cells was found to be decreased in the circulation of SLE patients compared to healthy individuals.

In summary, the work presented in this thesis shows how B cell responses are regulated in different autoimmune contexts. A protective role for B cell responses in atherosclerosis was found, as well as a novel co-receptor involved in the response to self-antigens and the memory response to apoptotic-cell derived lupus-related self-antigens has been characterized in more detail than ever before. These findings are important for the understanding of B cell regulation in autoimmunity and can be implemented to inhibit harmful and promote protective responses in therapeutic approaches to combat autoimmune disease.

# LIST OF SCIENTIFIC PAPERS

I. Grasset EK, Duhlin A*, Agardh HE*, Ovchinnikova O, Hägglöf T, Forsell MN, Paulsson-Berne G, Hansson GK, Ketelhuth DFJ, Karlsson MCI
**Sterile inflammation in the spleen during atherosclerosis provides oxidation-specific epitopes that induce a protective B-cell response**
*Proc Natl Acad Sci U S A, 2015, 112(16), E2030-38*

II. Duhlin A, Chen Y, Wermeling F, Sedimbi SK, Lindh E, Shinde R, Halaby MJ, Kaiser Y, Winqvist O, McGaha TL, Karlsson MC
**Selective memory to apoptotic cell-derived self-antigens with implications for systemic lupus erythematosus development**
*J Immunol, 2016, 197(7), 2618-26*
(Highlighted in Nat Rev Rheumatol 2016 Sep;12(10):559)

III. Duhlin A*, Grasset EK*, He C, Amara K, Sippl N, Lindh E, Vargas L, Dahlberg CI, Westerberg LS, Smith ECI, Malmström V, Pierce SK, Karlsson MC
**Scavenger receptor CD36 on B cells senses modified self-antigens to prevent autoimmunity**
*Manuscript*

*equal contribution

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AID | activation-induced cytidine deaminase |
| APRIL | a proliferation-inducing ligand |
| ApoE | apolipoprotein E |
| BAFF | B cell-activating factor of the TNF family |
| BCR | B cell receptor |
| Btk | Bruton’s tyrosine kinase |
| CSR | class switch recombination |
| DAMP | danger-associated molecular pattern |
| DC | dendritic cell |
| dsDNA | double stranded deoxyribonucleic acid |
| FcR | Fc receptor |
| FDC | follicular dendritic cell |
| FOB | follicular B cell |
| GC | germinal center |
| IC | immune complex |
| Ig | immunoglobulin |
| IL | interleukin |
| ILC | innate lymphoid cell |
| ITAM | immunoreceptor tyrosine-based activation motif |
| ITIM | immunoreceptor tyrosine-based inhibitory motif |
| JNK | c-Jun N-terminal kinase |
| LDL | low-density lipoprotein |
| LPS | lipopolysaccharide |
| MFG-E8 | milk fat globule-EGF factor 8 protein |
| MHC | major histocompatibility complex |
| MS | multiple sclerosis |
| MZB | marginal zone B cell |
| MZM | marginal zone macrophage |
| NFAT | nuclear factor of activated T cells |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |

| | |
|---|---|
| NKT | natural killer T |
| NLR | NOD-like receptor |
| NLRP3 | NOD-like receptor family, pyrin domain containing 3 |
| oxLDL | oxidized low-density lipoprotein |
| PALS | periarteriolar lymphoid sheath |
| PAMP | pathogen-associated molecular pattern |
| PBMC | peripheral blood mononuclear cell |
| PC | phosphorylcholine |
| PD-1 | programmed cell death protein 1 |
| PI3K | phosphoinositide-3-kinase |
| PLCγ2 | phospholipase C γ2 |
| PRR | pattern recognition receptor |
| PSR | phosphatidylserine |
| RA | rheumatoid arthritis |
| RF | rheumatoid factor |
| SHIP | SH2-domain-containing inositol polyphosphate 5' phosphatase |
| SHM | somatic hypermutation |
| SLE | systemic lupus erythematosus |
| SR | scavenger receptor |
| T1 | transitional type 1 |
| T2 | transitional type 2 |
| TCR | T cell receptor |
| TD | T cell-dependent |
| $T_{FH}$ | T follicular helper |
| TI-I | T cell-independent type I |
| TI-II | T cell-independent type II |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |
| Treg | regulatory T cell |
| wt | wild type |

# 1 INTRODUCTION

## 1.1 THE IMMUNE SYSTEM

Our immune system protects us from pathogens in our environment, such as bacteria, viruses and fungi. It is a complex network made up of physical barriers and specialized proteins, cells and organs that all work together to prevent pathogens from causing disease in the body. Once the immune system has successfully mounted an immune response upon intrusion of a pathogen it will remember it and can more effectively and rapidly fight a reoccurring intrusion of the same pathogen. This is called immunological memory and is one of the hallmarks of our immune system. Another crucial task for the immune system is to maintain homeostasis as well as be tolerant to the presence of the body's own structures and proteins, or so called self-antigens. A loss of this tolerance can lead to the immune system attacking cells and tissues of the body itself, causing autoimmune disease [1].

The various cells, receptors and other mediators that make up the immune system are typically classified as belonging to either the rapid and broader innate immune system or the slower but more specific adaptive immune system. It should however be stated that the two systems very much depend on each other and that there are for example immune cells that belong to the adaptive immune system but show innate features in the way they recognize and respond to antigen [2, 3].

### 1.1.1 Innate immunity

The separation between the innate part of the immune system and the adaptive is based a lot on antigen recognition. Immune cells of the innate immune system express receptors which are encoded in our germline DNA and therefore as opposed to in adaptive immune recognition do not require gene rearrangement. These germline encoded receptors recognize molecular patterns such as lipopolysaccharide (LPS), lipoteichoic acid and glycans and have been conserved through evolution to protect us from pathogens with these structures. These receptors are collectively termed pattern recognition receptors (PRR) because they recognize pathogen-associated molecular patterns (PAMP) [4]. PRRs can be membrane bound receptors but they can also be located in the cytosol and some are functional only when secreted. A family of essential PRRs are the Toll-like receptors (TLR) where some are membrane bound and some are intracellular and the various family members are categorized based on what class of PAMP they bind. The TLR that was first identified as a PRR was TLR4 for its ability to bind LPS; a major component of gram-negative bacteria. Intracellular TLR3, -7, -8 and -9 are instead located in the endosomal compartment where they can sense PAMPs of microbial nucleic acids such as single-stranded RNA and unmethylated CpG dinucleotides. TLR2 can only function upon heterodimerization with either TLR1 or -6 which could lead to an increased ligand specificity [5]. TLR heterodimers can also associate with other receptors and in this way facilitate binding and uptake of antigens [6]. This phenomenon is considered in more detail in section 1.5.

Another class of PRRs are the scavenger receptors (SR). They bind to a broad range of epitopes found on both microbes, modified lipids and apoptotic cells and as the name implies scavenge or clean the body of these antigens. As a group they are fairly diverse in structure

and are classified more based on their common function. In the context of innate immunity they play an important role in phagocytosis and cell adhesion [7]. For their ability to bind modified lipids and apoptotic cells they have also been implicated in the context of atherogenesis and autoimmunity respectively [8, 9]. A role for the class B scavenger receptor CD36 on B cells is the focus of paper III.

Oxidation is constantly occurring in nature and in our bodies. As a result of oxidative processes, oxygen reactive species are formed and these can in turn oxidize lipids, proteins and DNA creating so called oxidation-specific epitopes. Epitopes like this are, although being self-epitopes, recognized as damaged structures that could cause danger to the host if not taken care of by the innate immune system [10]. Oxidation-specific epitopes are recognized and bound by PRRs and have therefore come to be referred to as danger-associated molecular patterns (DAMP) and some of the epitopes also share molecular mimicry with PAMPs. An immune response triggered by DAMPs gives rise to what has been termed sterile inflammation as it occurs in the absence of pathogen [11]. The first example of a disease caused by sterile inflammation is gout, where hyperuricemia nucleate crystals of monosodium urate deposit in joints inciting an acute inflammatory response [12]. One of the oxidation-specific epitopes most studied is phosphorylcholine (PC) which is present on both oxidized lipids, apoptotic cells and also some pathogens such as *Streptococcus pneumoniae* [13]. Understanding the regulation of immune responses elicited against oxidation-specific epitopes is central to this thesis and is considered in more detail in section 3 and in the papers.

The cellular entity of the innate immune system is made up of monocytes, macrophages, dendritic cells (DC) and neutrophils belonging to the myeloid lineage of immune cells, as well as natural killer (NK) cells and innate lymphoid cells (ILC). Macrophages and neutrophils are phagocytic cells which after recognition of microbes by PRRs can phagocytose or engulf them and then destroy them in intracellular vesicles. Monocytes are abundant in the circulation and can upon inflammation migrate into tissues and further differentiate into macrophages. Dendritic cells are so called antigen-presenting cells (APC) as they present antigen to T cells and thereby form a very important bridge between innate and adaptive immunity. Although the DC is often appreciated for its antigen presenting capability it should be noted that also macrophages and B cells are APCs [1].

A consequence following binding of PRRs as well as an essential driver of inflammation in innate immunity is the activation of the inflammasome; a multiprotein complex present in myeloid cells. As described myeloid cells use PRRs to bind to and phagocytose pathogens, modified antigens and danger-associated ligands. Well in the cytosol the engulfed material can be further sensed by intracellular PRRs such as NOD-like receptors (NLR) and they can in turn bind the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain). Now the inflammasome is formed and can go on to cleave pro-caspase 1 into its active form caspase 1 and this enzyme can cleave pro-interleukin 1β (pro-IL1β) and pro–IL18 into the active cytokines IL-1 and IL-18 [14]. IL-1β is one of the most important cytokines to drive inflammation, both by recruiting other innate immune cells and activating vascular endothelium and lymphocytes [15]. Hence, activation of the inflammasome is crucial in regulating the inflammatory response elicited by the innate immune system. The inflammasome has also been shown to affect the subsequent adaptive

immune response as well as play a role in atherosclerosis, a mechanism that we could contribute more evidence to in paper I [16, 17].

There are some immune cells that are more difficult to categorize as belonging to the innate or adaptive immune system. That is because they express antigen receptors that have gone through somatic gene rearrangement, a hallmark of adaptive immunity. At the same time these cells have the ability to, or even preferably, respond to antigen in the first line of defense. Antigen receptors of these cells, although rearranged to some degree, are more conserved. Similar to PRRs of the innate immune system, they have to a greater extent affinity for ligands with PAMPs or DAMPs as opposed to conventional lymphocytes of the adaptive immune system. These so called innate-like lymphocytes are γδ T cells, natural killer T (NKT) cells, B1 cells and marginal zone B cells (MZB) [18, 19]. B cell regulation is central to this thesis and MZBs and B1 cells will be further described in section 1.2.

Innate immunity is the initiator of inflammation but when innate immune defenses are not sufficient to overcome the intrusion of a pathogen more specific effector cells are needed. Cues from the innate immune system are necessary for dictating an appropriate adaptive immune response to a particular pathogen.

### 1.1.2 Adaptive immunity

Adaptive immunity is as opposed to the inherent innate immune system and as the name implies something that develops during the lifespan of an individual as an adaptation to the intrusion of a specific pathogen. This is mirrored in the diversity and specificity of the antigen receptors of B cell and T cell lymphocytes; the major cellular components of adaptive immunity. This level of specificity however comes at a prize as adaptive immunity takes days to develop in contrast to the innate immune response which is initiated within minutes or hours. Activation of adaptive immunity is also very much dependent on the innate immune system and once activated, the adaptive immune system can in return potentiate innate effector mechanisms [1].

The B cell receptor (BCR) and T cell receptor (TCR) are membrane bound antigen-recognition receptors. They are part of the immunoglobulin (Ig) superfamily of proteins and encoded in the *Ig* loci of B cells or *tcr* loci of T cells [20]. The way that these receptors are assembled is unique as the ready receptor is encoded by the joining of different gene segments in somatic tissues in a process known as V(D)J recombination [21, 22]. In this process one variable (V), one joining (J) and sometimes one diversity (D) gene segment are joined together. The incredible diversity amongst these receptors as a result comes from both combinatorial diversity because of the immense number of different combinations of the three gene segments that can be formed, and also junctional diversity as the segments are joined in a non-absolute manner [23]. To accomplish this intricate recombination of different gene segments two crucial lymphocyte-specific proteins are needed, namely the RAG-1 and RAG-2 enzymes. They are specifically co-expressed in only B and T cells and are responsible for the actual cleavage of the gene segments [24].

As mentioned in section 1.1.1 the presentation of antigen by DCs to T cells is an important step in an immune response to activate the adaptive immune system. As opposed to the BCR which can bind free antigen the TCR can only bind to antigen peptides presented by a major

histocompatibility complex (MHC) molecule on the surface of an APC. MHC class I molecules present antigens processed in the cytosol and are recognized by CD8 T cells, while MHC class II molecules present antigens processed in endosomal compartments and are recognized by CD4 T cells. These molecules were first discovered for causing rejection of transplanted tissues because they were recognized as foreign by the recipient's immune system [25]. The binding of the TCR to the antigen-MHC complex is the first activating signal. The second signal is provided by co-stimulatory molecules and depending on the pathogen the third signal is provided by different sets of cytokines produced by the APC. These three signals will activate the T cell to undergo clonal expansion and differentiation into a number of effector T cell subsets. Which subset is governed by the nature of the antigen [26].

A B cell also gets its first activating signal from binding the antigen with its BCR. The endocytosed antigen is then displayed on the surface of the B cell in an MHC class II molecule. Unlike T cells, B cells are also APCs. The second signal is given by an antigen-experienced T cell that recognizes the antigen-MHC complex. T cells that provide this B cell help are called T follicular helper ($T_{FH}$) cells. They are CD4 T cells and specifically express the B cell follicle homing receptor CXCR5 [27]. A properly activated B cell can differentiate to become a germinal center (GC) B cell, a memory B cell or an antibody-producing plasma cell. B cells can also become activated without T cell help. Different pathways of B cell activation and B cell fates will be described in detail in section 1.2.3.

#### *1.1.2.1 Humoral immunity*

One of the most important effector functions of B cells in an immune response is to produce antibodies that can in turn neutralize pathogens, help phagocytes to recognize pathogen for engulfment and activate the complement system. An antibody is the soluble form of a BCR and consists of a constant region that determines its effector function and a variable region that determines the antigen-binding specificity. It has a two-fold axis of symmetry and has two identical heavy chains containing both the constant and variable region and two identical light chains containing only the variable region. The constant region of an antibody, also called Fc region, consists in five classes or isotypes; IgM, IgD, IgG, IgE and IgA. The BCR of a non-activated or naïve B cell is always of the IgM isotype but following activation the isotype can be switched to another isotype class with effector functions needed to better fight the pathogen in question. The regulation of isotype class switching is partly mediated by cytokines secreted from activated T cells and can be considered the third signal in B cell activation [1].

IgG antibodies can trigger effector responses such as macrophage phagocytosis, ADCC (antibody-dependent cell-mediated cytotoxicity) by NK cells, neutrophil activation and inhibition of B cell activation by immune complexes (IC) by binding to Fc receptors (FcR). As the name implies the antibody binds the receptor with its Fc region. Studies where FcRs were first identified showed that the binding was independent of the variable part of the antibody, the so called F(ab) region [28]. There are activating FcRs that signal through an immunoreceptor tyrosine-based activation motif (ITAM) and there is one inhibitory FcR; FcγRIIb, that signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM) [29]. Since FcRs are expressed on a variety of immune cells and can be both activating and

inhibitory they have the ability to regulate an immune response both in the innate and adaptive branch and play an important role in both clearing an infection but also in governing anti-inflammatory responses and tolerance [30].

#### *1.1.2.2 Immunological memory*

A hallmark of adaptive immunity is the creation of long-lasting protective immunity following the first encounter of a pathogen by the immune system. This phenomenon is called immunological memory as the immune system remembers the pathogen upon a second encounter and therefore can mount a faster and more efficient immune response compared to the primary encounter. This concept is the biological foundation in vaccine development where extensive research is being done on how to enhance and regulate both the cellular and humoral components of a memory response to develop potent vaccines [31, 32].

The major components of immunological memory are memory B and T cells and long-lived plasma cells which reside in the bone marrow where they constantly produce antibodies. They are actually contributing to a large fraction of the total amount of antibodies in the circulation and they can reside in the bone marrow for a lifetime [33]. Whether the long-lived plasma cell pool in the bone marrow is being continually replenished by memory B cells in the periphery or if its survival in the bone marrow is dependent upon a local survival niche is still being debated and there are some competing concepts [34]. In every recall response to a certain antigen the antibodies produced will be of higher affinity for the antigen as they have gone through more rounds of selection and this also adds to the increased efficiency of a memory response. Hence, the more times a pathogen is recalled the more efficient the immune system will be in combating it [35]. In paper II we investigate how some of these processes are similar but interestingly also differ in the memory response to a self-antigen.

### 1.1.3 The spleen

All immune cells develop from hematopoietic stem cells in the bone marrow, except for a specialized B cell subset that is derived from the fetal liver and neonatal bone marrow and so called tissue resident macrophages which are derived from the embryonic yolk sac [36, 37]. The thymus and the bone marrow are both classified as central or primary lymphoid organs because they are home to developing immature progenitors of immune cells. Although the thymus is the most important organ for T cell development, T cell progenitors also stem from the bone marrow. When cells leave the bone marrow they go to secondary lymphoid organs to continue their development or differentiation upon activation. Secondary lymphoid organs are lymph nodes, the GALT (gut-associated lymphoid tissue) and the spleen. Due to the types of responses investigated in this thesis this chapter will focus on the anatomy and function of the spleen in the immune system.

The spleen is divided into areas of red pulp and white pulp. The red pulp is so called because here is where blood is filtered through the spleen in a specialized structure of veins. Red pulp macrophages also make up this area and thereby have an ideal positioning for phagocytosing senescent erythrocytes, an important function of the spleen. The white pulp is the lymphoid compartment where the positioning of B cell follicles and T cell zones or periarteriolar lymphoid sheaths (PALS) makes for an excellent setup of B and T cell cross-talk and is quite similar to the structure in other secondary lymphoid organs [38].

The spleen also harbors a unique anatomical structure called the marginal zone which borders the red and white pulp. Here, specialized subsets of macrophages and B cells reside in an opportune location to capture and respond to blood-borne antigens, as the marginal zone is where blood is being filtered into the spleen through a sinusoid system to eventually enter the red pulp. Marginal zone macrophages (MZM) and marginal metallophilic macrophages are macrophage subsets specific to the marginal zone (Figure 1). In addition to their opportune location, they also express sets of PRRs that are well suited to bind the blood-borne antigens entering the marginal zone. The MZMs can provide the MZBs with antigen and MZBs are in turn specialized to respond to blood-borne antigens and can also transport antigen and ICs into the follicle and deposit it on follicular dendritic cells (FDC) [39, 40].

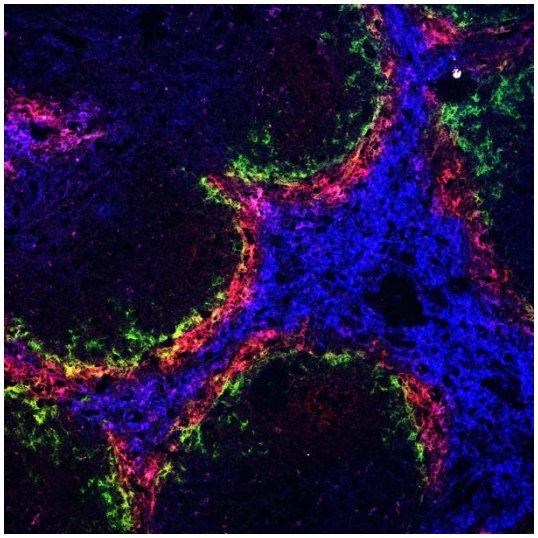

**Figure 1.** Histology of a mouse spleen showing the follicles surrounded by the marginal zone and the red pulp. Stained for MARCO – MZMs (red), MOMA1 – marginal metallophilic macrophages (green) and F4/80 – red pulp macrophages (blue).

## 1.2 B CELLS

B cells develop in the bone marrow but the B in B cell does not stand for bone marrow but for bursa of Fabricius, a lymphoid organ in chickens where B cells were first discovered back in 1965. This groundbreaking study by Cooper and colleagues established the B cells and T cells as separate lineages originating from either the bursa (B) or thymus (T). From their studies of irradiated chickens they could also attribute hallmark immune effector functions to either subset. The B cells were responsible for antibody responses and the T cells for cellular effector functions such as delayed-type hypersensitivity and graft-versus-host rejection [41]. The discovery has shaped the course of modern immunology and greatly contributed to the study of immunodeficiency conditions, cancer and autoimmunity. The B cell is classically considered as a cell belonging to the adaptive part of the immune system that requires T cell help to become activated and subsequently differentiate and produce antibodies to attenuate infection. But B cell biology is diverse and there are different B cell subsets that are located in different anatomical locations in the body and they differ in their antigen recognition properties and activation pathways. In this chapter the development and diversity of B cells and their activation during the course of an immune response will be described.

### 1.2.1 B cell development

The bone marrow is the primary location for early B cell development from stem cell to immature B cell and development from immature to mature B cell takes place in secondary lymphoid organs. Alternative locations for early B cell development are also the fetal liver and the lamina propria of the gut [37, 42]. Development in the bone marrow starts with the

early pro-B cell and consists of a number of steps during which V(D)J recombination takes place to assemble a functional BCR. In each step a gene rearrangement takes place to produce another protein chain of the BCR and successful rearrangement is basically the cue for moving on to the next stage. This process is tightly regulated to ensure the high specificity and thereby diversity of the resultant B cell repertoire, that each B cell only expresses BCRs with a singular specificity and also to avoid the production of B cells with high specificity to self-antigens, as this could cause autoreactive immune responses and as a result autoimmune disease. During V(D)J recombination, there is no control for the fact that the BCR generated from gene rearrangements will not be reactive towards self-antigens. There are however checkpoints both in the bone marrow and the periphery to ensure that B cells are tolerant to self-antigens [43].

As much as 75 % of early immature B cells have been estimated to display auto-reactivity. There are however control mechanisms at play to minimize the amount of self-reactive immature B cells exiting the bone marrow. The BCRs of immature B cells are tested for self-reactivity by the surrounding tissue in the bone marrow. If a B cell reacts to one of these antigens it can try to rearrange its light chains once again, a process known as receptor editing. A B cell can go through several rounds of receptor editing but if it has used up all its V-J segments and is still autoreactive, apoptosis is induced, a concept known as clonal deletion. A third mechanism is often induced when the self-antigen is only weakly cross-linking, as is the case for some small soluble proteins. This induces a state of unresponsiveness or anergy, which means the cell is viable and can still exit to the periphery but well there cannot be activated upon antigen encounter [44]. Together these mechanisms create so called central tolerance but in spite of this about 40 % of B cells leaving the bone marrow are still self-reactive. This makes sense though as not all antigens of the body can be presented in the bone marrow. Thankfully there are similar mechanisms in the periphery to induce tolerance. Also here, B cells can undergo clonal deletion or receptor editing upon encounter of a self-antigen or be induced to a state of anergy and these mechanisms are responsible for peripheral tolerance [45, 46]. About 20 % of peripheral mature B cells are however still autoreactive, although largely against cytoplasmic antigens which could be explained by the fact that they are less accessible for antigen-recognition during B cell development [43].

After V(D)J rearrangement and assembly of a functional BCR that has been tested for self-reactivity, B cells leave the bone marrow as transitional B cells, so called as they are transitioning from immature to mature B cells. When B cells get ready to leave the bone marrow they acquire an increased density of IgM on their surface and upon exit they also acquire the surface expression of IgD [47]. The transitional B cells home to the spleen where they can continue their development into fully mature B cells. There is some controversy regarding the developmental stages of transitional B cells into mature B cells, however they can be distinguished using different surface markers [48, 49]. The phenotype of these cells and other B cells will be described in the next section.

### 1.2.2 B cell subsets

There are transitional type 1 (T1) and transitional type 2 (T2) B cells. These are immature B cells that upon their exit from the bone marrow express IgM (the BCR) but as T1 B cells

differentiate into T2 B cells they acquire increased surface expression of IgD as well as other markers such as CD23 and CD21 [49]. Evidence has also been put forth supporting the existence of a third transitional B cell population, namely transitional type 3 (T3) B cells, which can be distinguished using an additional marker; AA4 [50]. Transitional B cells home to the spleen where they differentiate to mature B cells.

The mature B cells are divided into B2 and B1 cells. The B2 cells are further divided into either follicular B cells (FOB) or MZBs. FOBs are the most abundant type of B cells and are as the name implies mainly located in B cell follicles in secondary lymphoid organs, but they also recirculate. Their positioning in B cell follicles opposite to the T cell zone make them well suited to respond to protein antigens in a conventional T cell-dependent (TD) immune response. MZBs are non-recirculating and only reside in the unique niche that is the marginal zone of the spleen. Phenotypically, MZBs are characterized by expressing high levels of CD21, CD1d and the SR CD36 [51]. CD21 on MZBs can help them to trap ICs in the marginal zone and transfer these into the follicle to deposit them on FDCs [52]. The expression of CD1d is important in presenting lipid antigens to *i*NKT (invariant NKT) cells [53]. A special functional characteristic of MZBs is how they respond rapidly to blood-borne antigens and how they are able to do this without T cell help [54]. A much debated question when it comes to FOBs and MZBs is what determines whether a transitional immature B cell will develop into one or the other. So far some of the factors implicated in this fate decision are signaling through the BCR, Notch2, the B cell-activating factor of the tumor necrosis factor (TNF) family (BAFF) receptor and the nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [55, 56].

B1 cells are quite different from B2 cells. They are derived from the bone marrow but also from the fetal liver. B1 cell development in relation to B2 development is still not fully understood and there are two different hypotheses where one claims that they stem from distinct precursors and the other that they are derived from a common precursor [57]. B1 cells are located in the spleen but also constitute a large portion of immune cells in peritoneal and pleural cavities in the body. They are self-renewing and can be further divided into B1a and B1b cells. Their common phenotype when it comes to surface markers is $CD19^{hi}B220^{lo}CD43^{+}CD23^{-}IgM^{hi}IgD^{lo}$. The B1a subset is however distinguished from the B1b cells on the basis of also expressing CD5 [37]. Another major characteristic of B1 cells is their ability to produce natural antibodies which are antibodies that are produced without the presence of pathogens as they have been shown to be present in germ-free mice at steady-state [58]. It should however be noted that B1 cells are not the only source of natural antibodies, as MZBs are also capable of producing antibodies under homeostatic conditions [19].

There are also subsets of B cells with regulatory functions. It has been hard to reach a consensus on the phenotype of these cells and to date it is unknown how they are developmentally linked to each other and other B cell subsets. What is agreed upon though is the ability of these cells to produce the anti-inflammatory cytokine IL-10 [59]. Subsets that so far have been reported to do so are T2-MZP (transitional type 2 marginal zone precursor) cells, MZBs, plasmablasts and a $CD1d^{hi}CD5^{+}$ B cell subset which has also been shown to differentiate into antibody-secreting cells after IL-10 production [60-64]. In addition to the

type of subset, the mode of activation is also of importance for inducing IL-10 and ligands engaging the BCR and TLRs have been shown to elicit IL-10 production from B cells [65].

The majority of the work in this thesis is based on studies in mice and therefore the B cell subsets described so far are murine B cell subsets. However, I would like to in a simplified manner mention the main phenotypes of the human peripheral circulating B cell subsets investigated in paper III, with regard to surface markers. Human peripheral circulating B cell subsets can be divided into transitional immature, naïve mature and memory B cells. In humans, plasmablasts and MZBs can actually also be found in the circulation [66, 67]. Naïve B cells, memory B cells and MZBs can be divided by their differential expression of CD27 and IgD. CD27 is a marker for memory B cells but is not present on immature or mature B cells, except for MZBs. IgD can then be used to distinguish between memory B cells and MZBs, where memory B cells are $IgD^-$. There are a lot more specific surface markers for these subsets and the markers mentioned do for instance not distinguish between transitional immature and mature B cells [68].

Once activated, a B cell will differentiate into different activated B cell subsets which are the GC B cells, plasmablasts and plasma cells and memory B cells. Depending on the subset and antigen in question there are a number of different activation pathways for B cells to take, of which will be discussed in more detail in the following section.

### 1.2.3 B cell activation

The activation of B cells can occur with or without T cell help or in so called T cell-dependent (TD) responses and T cell-independent (TI) responses. The type of response depends a lot on the antigen in question and antigens can therefore also be classified as being TD or TI.

TI responses can be further divided into TI type 1 (TI-I) and TI type II (TI-II) responses. TI-I B cell activation is independent of engagement of the BCR and can be accomplished through TLR activation alone. A classic example is the binding of LPS to TLR4, something that has also been taken advantage of extensively in experimental research to activate B cells both *in vitro* and *in vivo*. TI-II responses on the other hand are dependent on engagement of the BCR, or rather cross-linking of numerous BCRs. This is accomplished by the fact that TI-II antigens often are long polysaccharides with many more or less identical antigenic sites. In a TD response the second signal is given by the T helper cell, but in TI responses help in form of a second signal for activation can come from other cells such as DCs, neutrophils and NKT cells [69]. And although T cell help is not essential in TI responses, T cells have been shown to play some role in regulating the response [70]. The innate-like B cell subsets MZBs and B1 cells are more prone to respond to TI antigens. Their BCR repertoire is rich in germ-line encoded specificities found on TI antigens, such as microbial carbohydrates, glycolipids as well as on self-antigens such as apoptotic cells [71]. Upon activation of their antigen, whether it’s a TI-I or TI-II antigen, these cells will rapidly differentiate into short-lived extrafollicular plasma cells [72]. The differentiation of MZBs and B1 cells into antibody producing plasma cells is the most abundant differentiation pathway for these types of responses. However, there is also some evidence to support that these cells can form abortive GCs and that TI-II immune responses generate memory B cells [73, 74].

TD antigens are protein antigens and although MZBs and B1 cells can participate in TD responses, they most commonly involve the activation of FOBs. The three signals required to activate a B cell in response to a TD antigen were briefly described in section 1.1.2. Once properly activated, the B cell can either enter the GC or directly differentiate to antibody producing plasma cells.

#### *1.2.3.1 The germinal center*

The GC is a specialized structure that appears in the follicles of secondary lymphoid organs during the course of a TD response. It consists of antigen-specific activated B cells that are undergoing clonal expansion and also manipulation of their BCRs to ultimately create B cells that are more efficient in clearing the pathogen in question [75]. When a B cell meets its antigen and is initially activated, it will upregulate CCR7 (chemokine receptor 7) and in response to CCL21 (chemokine ligand 21) migrate towards the T cell zone and in the T-B border is where GCs are formed. At this point the GC B cells upregulate an enzyme called activation-induced cytidine deaminase (AID), which together with other enzymes is responsible for somatic hypermutation (SHM) and class switch recombination (CSR) [76]. SHM is a process where random point mutations are introduced in the variable-region gene segments of the BCR and this results in B cells with altered affinity for the antigen. Since the mutations are random, both B cells with unchanged, higher or lower affinity for the antigen as well as self-reactive B cells can be generated. In order to select for and further expand only the B cells with sufficiently high affinity for the antigen, there is a specialized subset of cells in the GC called FDCs. Contrary to what the name implies, these cells are not DCs but were, when first discovered, mistaken to be because of their dendritic morphologic appearance. They are stromal in origin and unlike professional APCs they do not present antigen in an antigen-MHC complex but uses complement and FcRs for this purpose [77]. The presentation of antigen by FDCs to B cells in the GC reaction allows for antigen-driven selection and affinity maturation of the cells with the highest affinity for the antigen. The GC is divided into a dark zone, where proliferation and SHM takes place and following this B cells migrate to the light zone, where the FDCs are located and antigen-driven selection takes place. B cells that don't get selected undergo apoptosis and are phagocytosed by specialized macrophages called tingible body macrophages [78]. Defects in these macrophages have been linked to autoimmune disease, presumably because of the resultant accumulation of apoptotic cells [79]. CSR is the other AID-induced process that takes place in a GC and as the name implies means a switch of the isotype class of the BCR which will always, for a previously antigen-inexperienced B cell, be IgM and IgD. Different antibody subclasses have different effector functions and the choice of isotype in this process depends on the cytokine milieu induced by the pathogen. It should however be mentioned, that CSR has also been shown to occur independently of AID and the GC [80].

As described B cells need T cell help in a TD response to at all enter into a GC reaction, but also in the later stages of the reaction, there is a specific type of T cell that is extra helpful and essential for generating high-affinity antibody responses and B cell memory. These are $T_{FH}$ cells and they are a separate subset of T helper cells. They express CXCR5, programmed cell death protein 1 (PD-1), ICOS and CD40L and secrete the cytokine IL-21. The interaction of affinity maturation-selected B cells and $T_{FH}$ cells is the last checkpoint before the B cell leaves the GC and goes on to become a memory B cell or an antibody producing plasma cell.

However there is also a third option for the B cell, to re-enter the GC reaction and go through more rounds of SHM. The presence of $T_{FH}$ cells is important for the persistence of GCs. The lack of $T_{FH}$ cells aborts GCs, showing how essential of a checkpoint this specific T cell help is for further differentiation, but also for regulation of self-reactive GCs [79]. More specifically, a limiting role for $T_{FH}$ cells in GC B cell selection was also recently shown to be dependent on the amount of antigen presented on MHCII to the $T_{FH}$ cell which responds by secreting IL-4 and IL-21 [81].

#### *1.2.3.2 Plasma cells*

Upon activation, B cells can differentiate to antibody producing plasmablasts and plasma cells. Plasma cells have a slightly extended endoplasmic reticulum and a bigger cytoplasm than other B cells, as to maintain the production, storage and secretion of large quantities of antibodies [82]. They are characterized by their expression of CD138 and low expression of the pan B cell marker B220 and the expression of the transcription factor Blimp-1 [83].

In a TI response, activated B cells can move to medullary chords in lymph nodes and extrafollicular focis in the spleen to differentiate to plasmablasts and eventually plasma cells [84]. In a TD response the activated B cell can also take this route but can also enter the GC. GC B cells can further differentiate to long-lived plasma cells that home to the bone marrow where they can reside for a lifetime giving rise to antigen-specific antibodies [33]. Although this is where most of the long-lived plasma cells reside, a small population of long-lived plasma cells can also be found in the spleen [85]. Expression of CXCR4 by plasmablasts is important for the migration to extrafollicular sites and its ligand CXCL12 is indeed expressed in both extrafollicular focis, medullary chords and in the bone marrow [86]. The differentiation of activated B cells into plasmablasts at extrafollicular sites is associated with the loss of activation markers on the B cell's surface such as MHCII, co-stimulatory molecules, CD19 and the BCR itself. An important factor in extrafollicular focis for the maintenance of plasmablasts and their subsequent differentiation into bonafide plasma cells has been shown to be a specific DC population located in these sites that expresses high levels of CD11c [87]. In summary, the early adaptive immune response involving the activation of both FOBs, B1 cells and MZBs, whether it be TI or TD, is crucial to mount antibody responses that can specifically and efficiently combat the infection against a large variety of pathogens.

#### *1.2.3.3 Memory B cells*

Another outcome of B cell activation, following both a TI and TD response and in both the follicular GC and extrafollicular pathway, is the generation of memory B cells. As the name implies, the existence of memory B cells upon re-encounter of a pathogen is essential for the efficient development of neutralizing antibody responses [35]. The classical view of the phenotype of a memory B cell is that its BCR is isotype switched and of high affinity for the antigen, as it has been generated through CSR and SHM processes in a GC reaction. However, new evidence has emerged showing that there are also GC-independent memory B cells [88, 89]. So what governs the fate decision of an activated B cell to enter the GC or not and ultimately become a memory B cell as a result of either pathway? A couple of different regulatory events have been suggested. Competition experiments using high- and low-affinity

antigen specific B cells show that the early interaction with helper T cells could be of importance [90]. In more detail the SAP (signaling lymphocyte activation molecule-associated protein) has been shown to be important for the duration of T-B cell contact at this stage and could therefore also be involved in the fate decision [91]. CD40 signaling and regulation of Bcl6 by the cytokine IL-21 have also been implicated in affecting the choice of pathway [88, 92]. Recently, a study showed that memory B cells and long-lived plasma cells are generated at different points in time during the course of a GC reaction and even that memory B cells with different effector functions are generated at different time points [93]. Affinity for the antigen is also important for the choice between differentiation into a memory B cell or a long-lived plasma cell, where high-affinity B cell clones have a propensity for differentiating into plasma cells rather than memory B cells [94]. In addition to memory B cells developed from varying degrees of T-B cell interactions, memory B cells can also be generated from TI responses. B1 cells have been shown to generate memory B cells in a TI manner, although the recall response in this case, as compared to TD memory, was more qualitative than quantitative [95]. In summary, the quality and features of a memory response will vary depending on involvement of a previous GC reaction, the level of T cell help if any, the type of B cell activated and the type of antigen. The B cell memory response to self-antigens has been studied in much less detail and is something we provide more insight to with the findings in paper II.

#### *1.2.3.4 B cell signaling*

Events described so far concerning B cell activation have mainly been on a cellular level. However, like most cells, B cells have an intricate network of intracellular signaling proteins signaling in different pathways depending on what receptors that are engaged on the membrane surface. Pathways described in this section relates mainly to modes of B cell regulation investigated in paper III.

The first activating signal of a B cell is the binding of antigen to the BCR. It has in numerous studies been shown how important this is for the cell fate decision of a B cell. It is critical both at the earliest stages of development in the bone marrow, in the transition from immature to mature B cell, in GC selection and SHM processes and in reactivation of memory B cells [96]. The binding of antigen to the BCR activates the protein tyrosine kinase Lyn of the Src family of kinases. The BCR is coupled to the signaling components Igα and Igβ that contain ITAMs in their cytoplasmic tails that can get phosphorylated by Lyn. This in turn leads to the phosphorylation of another tyrosine kinase Syk, that can in turn activate the phosphoinositide-3-kinase (PI3K) leading to the phosphorylation and conversion of the signaling protein phosphatidylinositol biphosphate ($PIP_2$) to phosphatidylinositol triphosphate ($PIP_3$). The generation of $PIP_3$ recruits Bruton's tyrosine kinase (Btk) and phospholipase C γ2 (PLCγ2) to the membrane. This leads to a number of events such as the release of intracellular calcium and activation of downstream kinases and transcription factors NF-κB and nuclear factor of activated T cells (NFAT), which ultimately regulate the fate of the B cell [97]. The PI3K pathway and subsequent activation of the transcription factor FOXO1 has been shown to be the only pathway downstream of the BCR that is indispensable for B cell survival [98].

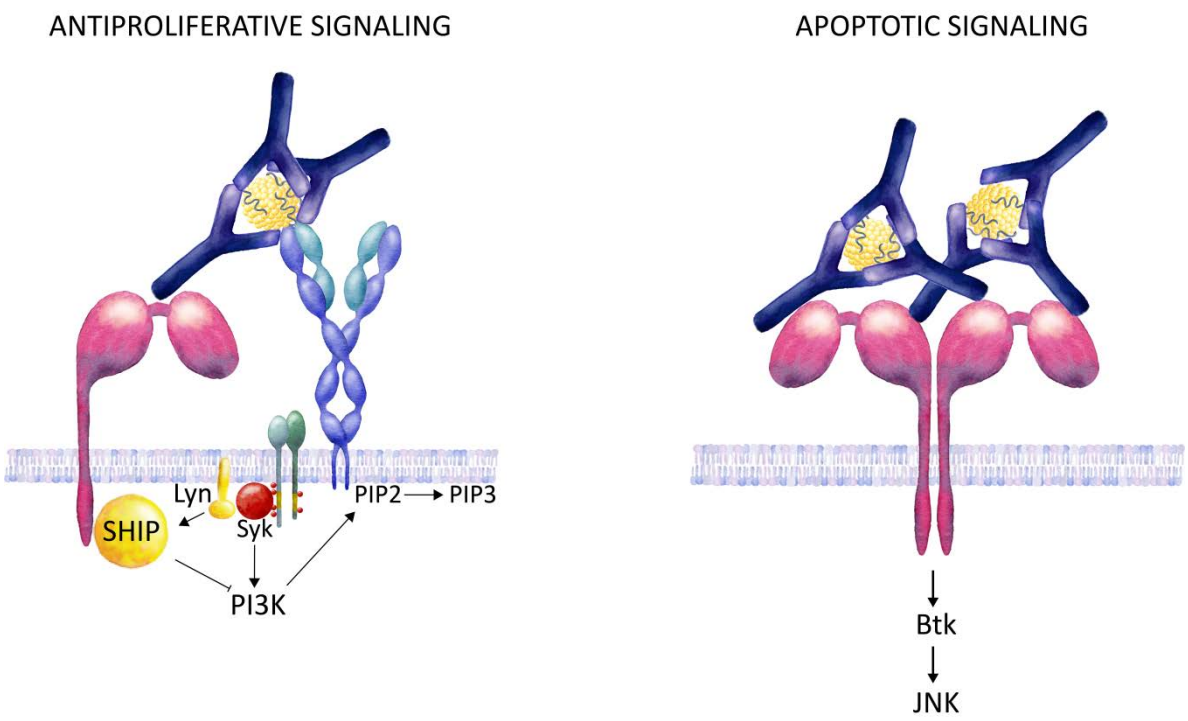


**Figure 2.** A simplified illustration of the two signaling pathways downstream of FcγRIIB in the response to ICs. On the left: On B cells carrying a BCR, engagement of the BCR and FcγRIIB by ICs leads to the activation of SHIP which will inhibit proliferative BCR signaling. On the right: On plasma cells, lacking a BCR, cross-linking of FcγRIIBs leads to the SHIP-independent activation of Btk and JNK which induces apoptosis.

Signaling events to inhibit B cell activation and as a result attenuate immune responses are of course also very important and are in principal mediated by in different ways interfering with the activating pathways described, downstream of the BCR. There are a number of inhibitory receptors on B cells such as CD22, PD-1 and paired immunoglobulin-like receptor B (PirB). However, the signaling downstream of FcγRIIB will be the focus of this chapter. FcγRIIB is the only FcR expressed on B cells [29]. FcγRIIB classically binds the Fc region of IgG ICs, which coligates the BCR with the antigen of the IC. Binding activates Lyn, which phosphorylates the ITIM of FcγRIIB. This in turn activates the SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP), which dephosphorylates $PIP_3$, causing Btk and PLCγ2 to dissociate from the cell membrane, which inhibits calcium flux and proliferation (Figure 2 - left). SHIP has also been shown to inhibit other proliferative pathways, such as the ones governed by the survival factor Akt and the MAP kinase [99]. With regard to the Akt pathway, it should however be mentioned that it has a complex role in cell fate decisions and repression of it can yield both inhibitory and activating consequences as a result of the subsequent B cell response [100]. FcγRIIB is expressed also on GC B cells and plasma cells. GC B cells that have undergone SHM can have lower affinity for the antigen and plasma cells have completely downregulated their BCR. This presents a new scenario for the binding of ICs to FcγRIIb, which affects the signaling downstream of the receptor due to the lack of BCR engagement. This cross-linking of FcγRIIB induces an apoptotic pathway that is independent of SHIP, Lyn, the ITIM, Syk and PLCγ2 but instead engages Btk and the c-Jun N-terminal kinase (JNK) (Figure 2 - right) [101]. Another SHIP-independent pathway, following cross-linking of only FcγRIIb has been proposed, where the involvement of the Abl (Abelson murine leukemia viral oncogene homolog 1) family kinase results in cell cycle

arrest and apoptosis [102]. Both of these pathways have implications for the selection and affinity maturation of B cells in the GC, to avoid the selection of B cells with low affinity or possibly self-reactive BCRs. Also at the plasma cell stage these pathways are of regulatory value to attenuate the antibody production and immune response when the presence of ICs have reached potentially harmful levels. Indeed, it has also been shown that mice lacking the FcγRIIB, and hence these pathways of regulation, develop autoimmune features spontaneuosly and even autoimmune disease on certain genetic backgrounds [103].

## 1.3 AUTOIMMUNITY

The first reference to the concept of autoimmunity was made by the German scientist and Nobel laureate Paul Erlich about a century ago when he coined the term *horror autotoxicus.* He described it as the immune systems's tendency to only attack foreign entities and to avoid attacking self [104]. However failures in the immune system, whether they are due to genetic factors or dysregulation caused by environmental triggers, can result in immune responses against self-antigens and ultimately autoimmune disease. The components of the immune system with most relevance to the work of this thesis have been described and they will now be further considered in the context of autoimmunity.

### 1.3.1 B cell regulation in autoimmunity

As described in this thesis, B cells have several essential roles to play in an immune response, such as antigen presentation, cytokine production, memory and antibody production. For the same attributes, they have also been shown to be critical in promoting a variety of autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), diabetes and multiple sclerosis (MS) [105-108]. B cell targeted depletion therapy with rituximab is currently approved for treatment of RA and is undergoing evaluation in clinical trials for the use as treatment also for MS and SLE patients [109]. Here, I will touch upon some of the factors that can influence the regulation of autoreactive B cell activation.

As described, there are both central and peripheral checkpoints to maintain self-tolerance. Weak cross-linking of the BCR by soluble proteins can lead to a state of anergy in the periphery. Immature anergic B cells cannot compete for entry into follicles and the marginal zone as normal B cells and usually have a half-life of only two to three days [110]. Factors that could influence the survival of these anergic self-reactive B cells are likely to play a role in autoimmunity. Transgenic mice that overexpress the survival factor BAFF suffer from autoimmunity with both circulating autoantibodies and glomerulonephritis caused by IC-deposition in the kidneys and older mice have also shown hallmark symtoms of the autoimmune disease Sjögren's syndrome [111, 112]. These mice also exhibit expanded pools of mature B cells and it is now known that the regulation of anergic self-reactive B cells in the periphery by BAFF is dependent on what stage of maturation the cell is in and that BAFF cannot rescue the cells that are more stringently deleted [113].

The dysregulation of various events in GCs and extrafollicular focis also contribute to autoreactive B cell responses. Some research carried out with the MRL.Fas$^{lpr}$ autoimmune-prone mouse strain crossed with mice transgenic for a BCR with affinity for the autoimmune

disease-related antigens DNA and rheumatoid factor (RF), actually show that the autoreactive response in this context bypasses the GC and takes place both in the T cell zone and in extrafollicular focis. There is also evidence supporting the fact that the plasmablasts in the extrafollicular focis have undergone isotype switch, somatic hypermutation and clonal expansion despite not having gone through the GC [114-116]. BAFF has as mentioned been implicated in autoimmunity and BAFF transgenic mice also display an expansion of plasmablasts at extrafollicular sites [111]. This highlights the importance of the extrafollicular pathway of activation in autoreactive B cell responses.

TLR7 and TLR9 bind RNA and DNA ligands respectively, which are also common autoantigens and these receptors have been shown to contribute co-stimulation to autoreactive B cell responses. Synergistic engagement of the BCR and TLR9 by IgG2a-chromatin ICs can activate autoreactive B cells [117]. The same type of activation could later be established also for co-engagement of RNA-associated autoantigens of the BCR and TLR7 [118]. TLR co-stimulation of B cells might even be more important than T cell help as they can induce both isotype switching and SHM in extrafollicular focis [119, 120]. The antigens mentioned are related to characteristic autoantibodies in SLE and these studies therefore provided new insight into why certain self-antigens might be preferred targets in this autoimmune disease.

DCs are important regulators of autoreactive B cell responses in a number of ways. They can present antigen to autoreactive T cells, which can in turn influence the B cell response. They also possess the ability to present non-degraded antigen directly to B cells, which can enhance humoral responses and it is possible that this could also affect autoantibody responses [121]. They are also producers of BAFF and a proliferation-inducing ligand (APRIL) which can directly enhance plasmablast proliferation and autoantibody production by promoting survival of self-reactive anergic B cells [122].

Regulatory IL-10 producing B cells have been found at elevated levels in autoimmune disorders and mice lacking these B cells develop more severe arthritis and experimental autoimmune encephalomyelitis (EAE) [59, 123]. Regulatory T cells (Treg), which also produce IL-10, can play a role in regulating autoreactive B cell responses. It has been shown that the absence of functional human Tregs leads to the accumulation of peripheral autoreactive B cells [124]. Further, B cell-specific deletion of IL-10 in mice leads to Treg deficiency, which in turn leads to the accumulation of pro-inflammatory T cells and exacerbated arthritis [125].

The importance of B cell-inhibitory receptors and signaling pathways was emphasized in section 1.2.3.4 and how the genetic deletion of FcγRIIB in mice leads to autoimmunity, largely due to the loss of the regulatory pathways described. In more detail, studies have shown that FcγRIIB is important for follicular exclusion of autoreactive B cells and also for regulating B cell activation by BCR-TLR co-ligation of ICs, a potent co-stimulatory mechanism of anergic autoreactive B cells alluded to earlier [126, 127]. The tyrosine kinase Lyn is involved in both activating and inhibitory signaling pathways in B cells. Because of the dual roles of Lyn its genetic deletion in mice have led to several observations of which one is the development of autoimmunity [128-130].

### 1.3.2 Systemic lupus erythematosus

SLE is in its true sense a systemic autoimmune disease as it can give rise to symptoms in most organs in the body. SLE has a low prevalence with only approximately 1 case in 2500 individuals in Northern Europe and 90 % of patients are women [131]. This supports a role for sex hormones in the pathogenesis of SLE but the mechanism behind this is still unclear [132, 133]. Patients can present with varying symptoms, all from rashes to anemia and psychosis. A characteristic of the disease is that symptoms arise suddenly, in so called flares, which are accompanied by periods of remission. Some of the most common known triggers of disease flares are UV-radiation and EBV infection [134]. How these disease flares relate to immune memory in the pathogenesis of SLE is the focus of paper II. Susceptibility gene loci have been identified for SLE and although they are important for the cause of disease, twin studies show that the concordance rate is fairly low. This indicates that environmental factors also play an important role for the etiology of SLE [135]. The presence of autoantibodies against self-antigens, such as double stranded DNA (dsDNA), in SLE patients is an important diagnostic marker. These autoantibodies form ICs with self-antigens and the ICs can settle in organs such as the kidneys and skin and the subsequent attraction of complement by the ICs causes local inflammation and tissue damage [136].

Apoptosis is a natural process that occurs in all tissues of the body. Although it occurs constantly, it is under normal conditions difficult to detect apoptotic cells in the blood or in tissues. However, they are found to a greater extent in SLE patients compared to healthy individuals [137, 138]. Autoantibodies with affinity for some of the antigens found on the blebbing membranes of apoptotic cells are present in SLE patients and there is considerable evidence supporting the fact that SLE patients have defects in the clearance of apoptotic cells. Some of the strongest evidence to support this is that the defect in some genes linked to apoptotic cell clearance can lead to SLE. Genes for which there is a strong link to disease development are encoding proteins that are in one way or another linked to apoptotic cell clearance. Examples are the complement component C1q, the phagocytosis enhancing Mer tyrosine kinase, the tingible body macrophage marker milk fat globule-EGF factor 8 (MFG-E8) and the apoptotic cell binding phosphatidylserine receptor (PSR) [139-142]. Further, exposure of mice to apoptotic cells in increasing amounts gives rise to autoantibodies against the antigens presented on the apoptotic cells and sometimes disease manifestations. This has been shown both with intravenous injection of apoptotic cells derived from various cell sources, as well as skin UVB irradiation [143-147]. In the model employed to study B cell regulation in systemic autoimmunity in the papers of this thesis, thymocytes were induced to a state of apoptosis with *in vitro* dexamethasone treatment. The repeated intravenous injection of these into wild type (wt) mice gives rise to autoantibodies that are also present in SLE patients [148]. The fact that an increased load of apoptotic cells generates an autoimmune response is somewhat peculiar, as apoptotic cell death is normally associated with an anti-inflammatory state [149]. This is true when apoptotic cells are successfully cleared from the body. However, a defect in clearance can lead to secondary necrosis of the apoptotic cells, a state where the cells still present apoptotic cell-derived self-antigens but that also promotes a pro-inflammatory response [150]. The combination of a pro-inflammatory milieu and the presence of modified self-epitopes on the apoptotic cells, which might not have been negatively selected against in central and peripheral checkpoints to the same extent as other

self-antigens, are likely to together be the cause of the transient autoimmune response in this model.

SLE patients have an increased risk of developing cardiovascular disease (CVD) [151]. Although the mechanisms underlying this fact are not fully elucidated, it is clear that normal risk factors for CVD are not enough to explain this correlation. Alterations in immune function related to autoimmunity are however much more plausible and have to a certain extent been proven. Immune activation and the role of B cells in atherosclerosis will be considered in the next chapter and relates to our findings in paper I.

## 1.4 ATHEROSCLEROSIS

CVD is one of the leading causes of death worldwide [152]. Atherosclerosis is the most common underlying cause of acute cardiovascular events, such as myocardial infarction and stroke. Lipid-rich lesions in large and medium sized arteries form atherosclerotic plaques. The plaques build up slowly over decades and when a rupture of their cap structure occurs, it causes thrombosis and occlusion of the vessel, which leads to the often fatal cardiovascular events [153]. One of the major risk factors for CVD is high plasma cholesterol levels. Although lifestyle changes and pharmacological approaches to lower lipid levels has improved patient outcome and reduced mortality, CVD remains to be one of the major causes of death [154]. The involvement of the immune system in the pathogenesis of atherosclerosis is indisputable when looking at the cellular composition of atherosclerotic plaques, and atherosclerosis is considered as an inflammatory disease [155]. The increased risk of developing atherosclerosis in diseases like SLE, RA and psoriasis points to an important role for autoimmune regulation elements in the disease [151, 156, 157].

Infiltration of low-density lipoprotein (LDL) in the vessel wall is the initiator of plaque formation. When LDL accumulates in the subendothelial space of the vessel wall it can be modified by oxidative processes to form oxidized LDL (oxLDL). The lipid accumulation also causes the endothelial and smooth muscle cells to upregulate adhesion molecules and produce chemokines that will attract monocytes which can be retained by the adhesion molecules and further stimulated to differentiate into macrophages by M-CSF (macrophage colony-stimulating factor) and GM-CSF (granulocyte-macrophage colony-stimulating factor), also produced by the endothelial cells [158]. Both monocytes and macrophages express PRRs that can bind variants of the lipids in the plaque. The scavenger receptors CD36 and SR-A (scavenger receptor class-A) on macrophages efficiently engulf lipids, causing the macrophages to eventually become lipid laden foam cells, the hallmark cellular component of atherosclerotic plaques [159]. Macrophages also express TLRs that can bind oxLDL, more specifically TLR2 and TLR4 have been shown to be activated by oxLDL. Interfering with the signaling pathway downstream of TLRs has been shown to reduce atherosclerosis, implying a pro-atherogenic role for these receptors [160-162]. Another innate sensor of oxLDL is the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome which can be directly activated by cholesterol crystals [17]. The pro-inflammatory role of the inflammasome with production of IL-1β and IL-18 has been linked to the initiation and progression of atherosclerosis, as these cytokines cause enhanced vascular inflammation and

increase plaque instability [163, 164]. In apolipoprotein E deficient (*Apoe*$^{-/-}$) mice, that develop hypercholesterolemia and atherosclerotic plaques spontaneously, IL-18 has been found at elevated levels and the lack of IL-18 in these mice was associated with a protective effect against disease development [165]. A similar protective effect was observed in *Apoe*$^{-/-}$ mice lacking IL-1β [166]. This finding was replicated in LDL receptor deficient (*Ldlr*$^{-/-}$) mice, a mouse model of atherosclerosis where the mice are maintained on a high-fat diet. Reduced atherosclerosis development was observed when bone marrow derived cells lacked NLRP3 and IL-1β [17, 166].

T cells are also a significant cellular population in the atherosclerotic plaque and a role for adaptive T cell immunity in the pathogenesis of atherosclerosis is well established [167]. In *Apoe*$^{-/-}$ mice completely lacking B and T cells the development of atherosclerosis is significantly reduced and the transfer of CD4 T cells aggravates disease, suggesting an overall pro-atherogenic role for T cells [168]. Th1 responses have in numerous studies been shown to drive plaque development and this has been much attributed to their capability of producing IFNγ (interferon γ) which can activate monocytes, macrophages and DCs [169]. In experiments with *Apoe*$^{-/-}$ mice, the lack of IFNγ attenuates disease while the exogenous addition of it aggravates plaque formation [170, 171]. Tregs on the other hand have been shown to counteract the disease, promoting inflammation. This has been demonstrated by aggravated disease development when Tregs are depleted and the protective effect of Tregs has been associated with their capability to produce the anti-inflammatory cytokines TGF-β (transforming growth factor-β) and IL-10 [172-174].

### 1.4.1 B cells in atherosclerosis

The role of B cells in the pathogenesis of atherosclerosis has not been as extensively studied as the contribution of T cell mediated immunity and research so far has been contradictory. However relatively recent studies has shed some more light on how different B cell subsets in different ways contribute to disease outcome [175]. Unlike T cells, B cells are only present in the actual plaque at low numbers but are more abundant in so called tertiary lymphoid structures present in the adventitia surrounding arteries and could potentially from this site influence inflammatory processes underlying the disease [176]. The importance of B cell-mediated humoral immunity in atherosclerosis has been appreciated for some time. The innate-like B1 cells and MZBs are sources of natural antibodies with an inherent reactivity for self-antigens, including oxidation-specific epitopes found on both oxLDL and apoptotic cells. An oxidation-specific epitope that has gained much attention in research related both to autoimmunity and atherosclerosis is PC. The natural antibody clone T15 binds to PC on both *S. Pneumoniae*, apoptotic cells and oxLDL, providing an interesting link between infection, autoimmunity and atherosclerosis. Several studies in mice have shown a correlation between anti-PC antibody responses and positive disease outcome [177-180]. In the studies alluded to, IgM antibody responses have mediated an atheroprotective effect. The role of IgG in atherosclerosis is however more unclear. IgG antibodies are present in atherosclerotic plaques and anti-oxLDL IgG antibody responses have been correlated with both positive and negative disease outcome [181, 182]. The role of IgG in atherosclerosis is further complicated by the fact that it binds to activating and inhibitory FcRs on cells both of the innate and adaptive immune system, making their direct effect more difficult to pinpoint [175].

Some of the more convincing evidence supporting an atheroprotective role for B cells comes from a study where *Apoe*$^{-/-}$ mice were splenectomized and then received adoptive transfers of either B or T cells. Only B cell transfers protected from plaque development. Splenectomized mice developed more severe disease than did sham-operated mice, which is also in line with data showing that splenectomized humans have an increased risk of developing CVD [183, 184]. Furthermore, *Ldlr*$^{-/-}$ mice fed a high-fat diet that received B cell-deficient bone marrow exhibited increased plaque formation compared to controls [185].

The protective role of B cells is however complicated by the partial attenuation of atherosclerosis that anti-CD20 induced B cell depletion has resulted in, in both *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice [186, 187]. Anti-CD20 treatment only effectively depletes B2 cells, which could suggest a protective role of B1 cells. BAFF is crucial for the maturation of B2 cells and BAFF-deficient *Apoe*$^{-/-}$ mice that almost completely lack B2 cells, but has an intact B1 compartment, are protected from atherosclerosis [188, 189]. Contrary to this, it has been shown that adoptive transfer of splenic B2 cells from *Apoe*$^{-/-}$ mice to B cell deficient *Apoe*$^{-/-}$ mice, kept on a high-fat diet, was atheroprotective and that the protection was dependent on the transcription factor Id3 (inhibitor of differentiation-3) [190]. Out of the two B1 cell subsets most of the atheroprotective effects have been linked to the B1a cells and the role of B1b cells in atherosclerosis remains unclear. B1 cells are the main source of natural antibodies and it has been shown that the transfer of B1a cells from sIgM$^{-/-}$mice, which express but do not secrete IgM, did not confer atheroprotection into splenectomized recipients like wt B1a cells did [191]. B1a cells are also the main producers of the anti-PC T15 antibody clone that has been linked to atheroprotection [177]. Splenectomy reduces the number of B1 cells in the spleen, which could explain why splenectomy aggravates disease [192]. However, splenectomy also removes the marginal zone. MZBs are also producers of natural antibodies and their role in atherosclerosis, compared to FOBs which is the much more abundant B2 cell, needs to be further elucidated. Another B cell subset whose role is yet to be established in atherosclerosis is the B regulatory cell. Although an atheroprotective role has been established for Tregs, recent studies on Bregs have come to conflicting results regarding the role for this elusive subset in atherosclerosis [193, 194].

## 1.5 CD36

CD36 is a scavenger receptor expressed on a wide range of cells both in and outside of the immune system [195]. As most PRRs it is well conserved and has multiple orthologs in other species [196]. Being a scavenger receptor, CD36 recognizes a variety of ligands, such as lipid and lipoprotein components of bacterial cell walls, β-glucans on fungi, erythrocytes infected with falciparum malaria as well as self-antigens such as apoptotic cells and oxLDL [6, 197-200]. For its ability to bind and internalize oxLDL, CD36 plays an important role in the pathogenesis of atherosclerosis [201]. Ligand binding to CD36 can result in several outcomes depending on the context, such as internalization and phagocytosis, pro-inflammatory responses and integrin activation. This is accomplished through various intracellular signaling events. Different signaling molecules and pathways have been implicated and they all involve Src family kinases and serine/threonine kinases of the MAPK family. In macrophages and platelets, Lyn and JNK are commonly involved in effector signaling, but in general the

signaling initiated by CD36 ligand binding seem to depend greatly on the cellular context [202-204]. In macrophages, CD36 can interact with both TLRs, TLR-heterodimers, tetraspanins and β integrins to mediate ligand uptake and intracellular signaling. CD36 was recently shown to internalize oxLDL through association with $\beta_1$ and $\beta_2$ integrins and tetraspanins CD9 and CD81, which in turn lead to phosphorylation of Syk and SHIP [205]. CD36 has also been shown to associate with the TLR heterodimers TLR1-TLR2, TLR2-TLR6 and TLR4-TLR6 and downstream signaling events involve pathways including both JNK and Lyn [206-208].

CD36 was only recently appreciated for being expressed also on B cells and interestingly with a preferentially higher expression on MZBs compared to other B cell subsets. Higher expression of CD36 could also be seen on unswitched plasma cells in the TI response to *S. pneumoniae,* as compared to switched $IgG1^+$ plasma cells. *S. pneumoniae* immunization of CD36 deficient (*CD36*$^{-/-}$) mice resulted in lower levels of plasma cells and an impaired humoral response as compared to wt mice [51]. Another study has shown that *CD36*$^{-/-}$ mice infected with *Leishmania major* showed higher levels of specific IgG, smaller lesions and faster recovery after infection. In the same study, CD36 expression was found to be dependent on the transcription factor Oct-2 in B cells but not in DCs or macrophages [209]. In humans there is so far only one study that has reported CD36 expression on a fraction of $CD19^+$ neoplastic B cells in patients with chronic lymphocytic leukemia [210]. In paper III a role for CD36 on B cells in the autoimmune response to apoptotic cells is investigated.

# 2 AIM

The overall aim of my thesis was to study how B cell responses are regulated in systemic autoimmunity.

The specific aims were:

Paper I – To characterize the atheroprotective B cell response in the spleen and how it could be induced.

Paper II – To investigate how memory to apoptotic cell-derived self-antigens develops and how it relates to SLE pathology.

Paper III – To investigate the role of scavenger receptor CD36 on B cells in the autoreactive immune response to modified self-antigens.

# 3 RESULTS AND DISCUSSION

## 3.1 A PROTECTIVE ROLE FOR B CELLS IN ATHEROSCLEROSIS

The immune system plays a crucial role in the pathogenesis of atherosclerosis and B cells have been shown to have dual roles when it comes to good or bad outcome of the disease. In a study where *Apoe*$^{-/-}$ mice were splenectomized and then received a transfer of either B cells or T cells, it was shown that splenectomy aggravated disease and that only the B cell transfer conferred protection from developing atherosclerosis [183]. This proved that the spleen and in particular the splenic B cells were important for protective immunity. In paper I we wanted to further characterize the protective B cell response originating in the spleen, focusing on the different B cell subpopulations present there. In addition to this we know that the marginal zone of the spleen is an anatomical niche for trapping antigens carrying oxidation-specific epitopes and antibodies raised against these epitopes, as for example anti-PC antibodies, have been associated with protection from atherosclerosis [148, 179]. Therefore we also investigated the effects of immunizing wt and *Apoe*$^{-/-}$ mice with apoptotic cells carrying oxidation-specific epitopes and investigated how this affected the B cell response as well as the disease outcome.

We investigated the frequency of different splenic B cell subsets in young non-atherosclerotic and aged atherosclerotic *Apoe*$^{-/-}$ mice as well as wt controls of both ages to get an indication as to which subsets could be of greater importance for the development of disease. We found that the precursor T1 and T2 B cell subsets both generally decreased with age in both strains. For the naïve B1 and B2 B cells in the spleen the most pronounced difference when comparing young and old *Apoe*$^{-/-}$ mice was observed in the MZB population, which was significantly increased in old *Apoe*$^{-/-}$ mice. Lastly, when investigating activated B cell subsets in the spleen of the same mice, we found significantly increased levels of both GC B cells and antibody-forming cells (AFC). This showed that there is an ongoing spontaneous activation in the spleen of aged *Apoe*$^{-/-}$ mice (Figure 3). When mice and humans age there is a reduced output of B cells from the bone marrow, which is compensated for by expansion in the periphery [211]. In the aged *Apoe*$^{-/-}$ mice we found reduced levels of bone marrow-derived T1 precursors together with an expansion of MZBs as well as an adaptive B cell activation. This prompted us to investigate the clonality of the expanded B cell subsets in aged *Apoe*$^{-/-}$ mice compared to wt controls. Using the method of spectratyping we could expand the VDJ region of different heavy chain variable region (Vh) families. In the Vh5 and Vh7 families the pattern of clonal expansion differed between *Apoe*$^{-/-}$ and wt mice showing that certain clones were preferentially expanded in the *Apoe*$^{-/-}$ mice. Interestingly, these Vh families are both known to harbor recombination combinations giving rise to anti-PC reactivity. In line with this, we could also detect increased levels of antibodies towards PC as well as oxLDL in the atherosclerotic *Apoe*$^{-/-}$ mice, confirming that there is actually an expansion of antibodies with the types of reactivities belonging to the Vh5 and Vh7 families. This suggested that expansion was driven by disease and not age.

Atherosclerosis is characterized by an increased amount of circulating lipids as well as oxidized lipids. These are constantly being filtered through the marginal zone of the spleen, where they can be bound by both MZM and MZB. This made us curious as to whether lipids could actually accumulate also in the spleen, as they do in vessel walls, to locally drive the

immune response. Indeed, we found lipid accumulation in red pulp macrophages in the spleens of aged *Apoe*$^{-/-}$ mice. We then hypothesized that these lipids could drive inflammasome activation in the spleen, since cholesterol crystals have been shown to directly activate this multiprotein complex and that this activation subsequently has implications for atherogenesis [17]. Inflammasome activation can be measured by caspase 1 activity and we found increased levels of caspase 1 in splenic macrophages and neutrophils in aged *Apoe*$^{-/-}$ mice. Furthermore, we could also induce inflammasome activation in the same types of cells by injecting wt mice intravenously with either oxLDL or apoptotic cells, which present the same epitopes found on for instance cholesterol crystals. We could for the first time show that during atherosclerosis, lipids accumulate not only in the plaque but also in the spleen and cause inflammasome activation and caspase 1 activity in macrophages and neutrophils. The subsequent release of IL-1β from these cells could potentially work as an adjuvant for the B cell response originating in the spleen [212].

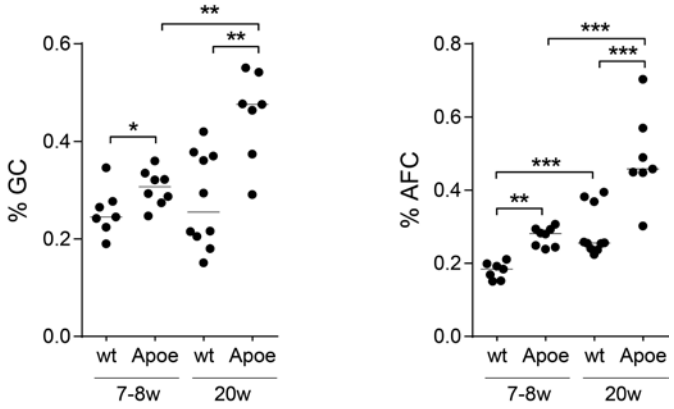


**Figure 3.** Frequency of GC B cells and antibody-forming cells (AFCs) determined by flow cytometry. Both subsets are increased in the spleens of old *Apoe*$^{-/-}$ mice compared to wt controls. n=7-10.

It has already been shown that immunizing mice with various foreign antigens containing PC-epitopes can be atheroprotective [179]. Here, we immunized young *Apoe*$^{-/-}$ mice repeated times with self-antigens in the form of apoptotic cells, which carry oxidation-specific epitopes such as PC, to test whether this could induce a B cell response similar to the spontaneous ongoing B cell response already seen in the old atherosclerotic *Apoe*$^{-/-}$ mice. The apoptotic cells would be injected intravenously, get trapped in the marginal zone of the spleen and induce inflammasome activation, which we hypothesized would augment the B cell response originating there and we now wanted to investigate whether this could also be beneficial for the outcome of atherosclerosis development. Strikingly, *Apoe*$^{-/-}$ mice that had received apoptotic cell injections during the course of disease development, showed significantly smaller plaques in both the aortic root and arch compared to non-injected controls. This was also accompanied by a drop in serum cholesterol levels (Figure 4). In order to further show that the protective effect was specifically contributed by the B cell response, we performed the same experiments in *Apoe*$^{-/-}$ mice crossed to the B cell-deficient μMT mouse strain. There was no difference in the severity of atherosclerosis with regards to either plaque formation or serum cholesterol levels in apoptotic cell-injected *μMTApoe*$^{-/-}$ mice compared to non-injected controls. The protective effect of the apoptotic cell injections was consequently B cell-dependent.

We also investigated the B cell response following apoptotic cell injections in *Apoe*$^{-/-}$ mice compared to wt controls. There was no increase in MZBs in *Apoe*$^{-/-}$ or wt mice following apoptotic cell injections, however there was a significant increase in GC B cells and AFCs in both groups of mice following injections. Injections of apoptotic cells were also given to NOD-like receptor family pyrin domain containing 3-deficient (*Nlrp3*$^{-/-}$) mice, which lack functional inflammasomes, and they failed to mount an anti-PC antibody response, suggesting that additional induction of the protective B cell response from the inflammasome is needed.

Paper I describes the splenic B cell response in atherosclerosis and pinpoints specific immune cells and regulatory pathways that are important for the development of protective immunity against the disease. It also shows how the protective response can be induced and highlights important similarities that the induced response has with the one in spontaneous disease. Finally, it establishes the importance of how immune activation in atherosclerosis partly originates in the spleen and this link will be valuable for future research.

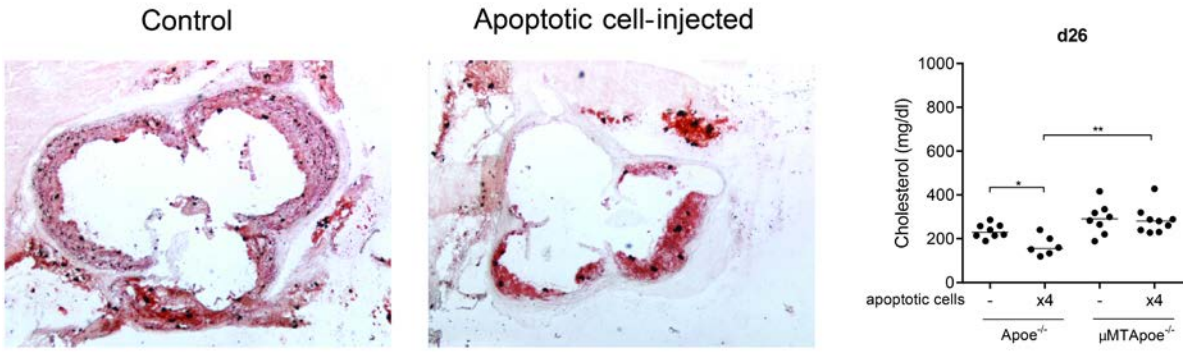


**Figure 4.** Representative pictures of lesions in the aortic root stained with oil red o. Apoptotic cell-injected *Apoe*$^{-/-}$ mice show reduced lesion size as compared to uninjected controls. n=6-7. On the right, cholesterol levels measured at day 26 using an enzymatic colorimetric assay. *Apoe*$^{-/-}$ mice show lowered cholesterol levels after apoptotic cell injections while there is no difference in *µMTApoe*$^{-/-}$ mice. The *µMTApoe*$^{-/-}$mice also exhibited higher cholesterol levels after apoptotic cell injections than did *Apoe*$^{-/-}$ mice. n=6-9.

## 3.2 IMMUNOLOGICAL MEMORY TO APOPTOTIC CELL-DERIVED SELF-ANTIGENS

Immunological memory is one of the most fundamental features of the immune system and a crucial mechanism to rapidly and efficiently clear recurring infections. The basic concept is that antigen specific long-lived memory B and T cells are formed after a primary infection and being already antigen experienced, they are then able to more rapidly respond to and clear a secondary infection with the same pathogen [31]. However, if the antigen in question is a self-antigen, as is the case in autoimmune disease, the memory response becomes more complex, since a self-antigen will not appear instantly like a pathogen but is more or less always present. Patients with the autoimmune disease SLE suffer from so called flares, which are sudden exacerbations of disease activity followed by periods of remission. These flares are often brought on by environmental triggers such as UV light, drugs and viral infections [213]. This kind of quick immune activation, shortly after being exposed to triggers that could enhance the presence of self-antigens, is similar to what would happen as a

consequence of immunological memory activation. In paper II we wanted to investigate the involvement of autoreactive immune memory in SLE pathogenesis in the context of B cell responses to apoptotic cells. We wanted to explore in more detail features of the memory response such as longevity, affinity maturation and specificity; aspects of autoreactive immune memory that remain relatively unexplored.

To study autoreactive immune memory we used the same model as in paper I and injected wt mice intravenously with syngeneic apoptotic cells to break tolerance to the self-antigens present on these cells. As expected, the mice developed increasing titers of both anti-DNA and anti-PC IgG over time. This response is however transient and about a month after the last apoptotic cell injection the autoantibody titers almost returned to baseline. Another month after this, we gave the mice a single boost injection of apoptotic cells in an attempt to recall the primary response. Indeed, the boost injection of apoptotic cells led to an increase in both anti-DNA and anti-PC antibodies and the response was both specific and quick, much like in classical immune memory (Figure 5). Autoantibodies can form ICs that in turn can accumulate in small blood vessels causing tissue injury and organ dysfunction [214]. We went on to test whether the autoantibodies in the memory response, as compared to antibodies from pre-immune (pi) serum or from the primary response, could be more pathogenic and thereby lead to kidney damage in the mice. In kidneys of mice having received the first boost injection there was little sign of Ig deposition. However, we also performed experiments where mice were subjected to a second boost injection of apoptotic cells about one month after the first boost. In the kidneys of these mice there was clear Ig deposition and the glomerular architecture was also altered, indicating kidney damage, possibly due to IC accumulation and increased complement activation [214]. Serum from mice having received the second boost also showed positive anti-nuclear antibody (ANA) staining with different staining patterns. No ANA-reactivity was detected in serum from mice that had only received the first boost. These experiments show a clear memory response against modified self-antigens found on apoptotic cells. The response is rapid as well as specific, since control experiments using a TD antigen, either in combination with apoptotic cells or without, showed no difference in the subsequent TD response. The memory response to the self-antigens also had pathological features but interestingly only in the second recall response, indicating that the memory response contains steps of immune activation that lead to increased pathology.

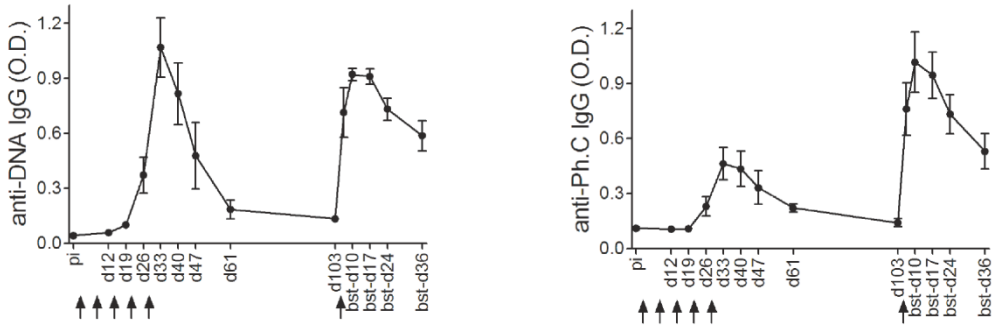


**Figure 5.** Serum levels of anti-DNA and anti-PCIgG measured by ELISA. Wt mice were administered apoptotic cells at the time points indicated by arrows. Increasing autoantibody titers was observed in the primary response and a rapid increase after the first boost injection.

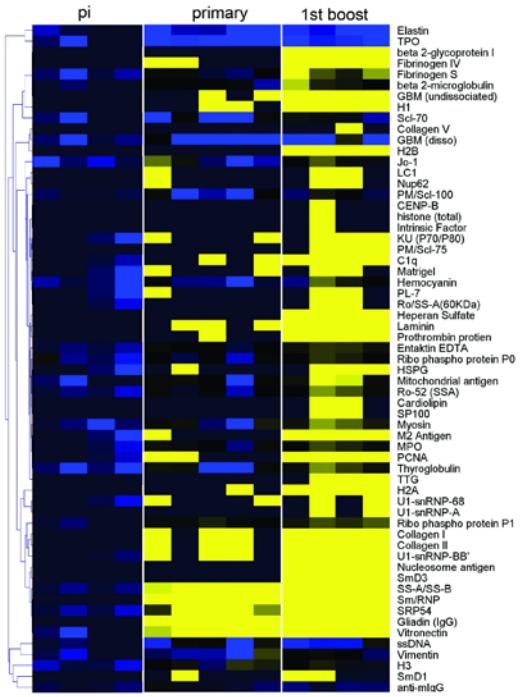


**Figure 6.** Serological spectrum of IgG autoreactivity in pi, primary and the first boost response investigated using an autoantigen microarray. The heat map shows the reactivity to 61 autoantigens meeting the minimal normalized fluorescence intensity requirement. The signal intensities are depicted on a relative scale. Blue, black and yellow represent Ag reactivity intensities below, close to and above the mean, respectively. Ag reactivity clusters are indicated at the left edge of the heat map. This array shows that the memory response against apoptotic cell-derived self-antigens is selective.

In a memory response, the antigen specific memory B cells undergo further subclass switching and SHM leading to more switched memory B cells and antibody responses with higher affinity for the antigen. Other cells of the adaptive and innate immune system such as $T_{FH}$ cells and DCs are also involved in these processes [215]. Using the same model, we further characterized the different players that could contribute to the memory response to modified self-antigens. In the spleen of mice having received the first or the second boost of apoptotic cells, compared to pi or the primary response, we found elevated levels of IgG2a- and IgG2b-switched plasma cells, GC B cells and $T_{FH}$ cells. In addition, we could in histology spleen sections see a striking expansion of $IgG^+$ plasma cells in extrafollicular foci. A majority of these could also be identified as PC-reactive since they stained positive for the T15-clone, which is the prototypic anti-PC clone [216]. This data shows that the memory response against apoptotic cell-derived self-antigens contains many of the hallmarks of adaptive immunity.

We performed a classic transfer experiment to further verify that the memory response we saw was real and specific to the self-antigens of interest. Splenocytes from mice that had received the primary immunization of apoptotic cells or splenocytes from pi mice were transferred to irradiated recipients that were then boosted with a single injection of apoptotic cells or given no injection. The boost injection elicited an autoreactive memory response only in the mice that had received self-antigen experienced splenocytes and again we could show that this memory response contained all the elements of a classical adaptive immune response as previously characterized.

Apoptotic cells are complex antigens and present a range of epitopes on their blebbing and budding plasma membrane [217]. Apart from the antigens we had already looked at, we therefore wanted to investigate other possible reactivities relevant to apoptotic cells and autoimmunity and what the reactivity pattern and intensity would look like in a primary response to apoptotic cells as compared to the memory response. To help us with this question we used an array with 61 different autoantigens [218, 219]. Sera from both pi as well as mice that had received either the primary or first boost immunization were tested for the autoantigen reactivities. Interestingly, as much as 19 of the autoantigen reactivities turned out to in varying degrees be enriched in the sera from the memory response (Figure 6). The

strongest response was detected for the Sm/RNP antigen, which is actually one of the anti-nuclear antigens with most clinical significance, as IgG-titers with this specificity are used as a diagnostic criterion for SLE [220].

Antibody responses are regulated through both positive and negative feedback mechanisms that are dependent on FcRs [29]. In a memory response, where antigen-specific antibodies are already present upon re-encounter of the antigen, the FcR-dependent feedback mechanisms are also of importance for the efficiency of the response. We wanted to study these mechanisms in the memory response to self-antigens. Apoptotic cells were coated with serum from either pi mice or mice that had received the primary or first boost immunization. IgG antibodies from first boost serum showed most efficient opsonization of the apoptotic cells and in line with this, first boost serum-coated apoptotic cells were preferred targets of phagocytosis by macrophages *in vitro*. In *in vivo* experiments, where mice were injected with serum-coated apoptotic cells from the same groups as mentioned and the subsequent antibody and B cell responses were measured, we found that mice immunized with boost-coated apoptotic cells had a stronger response. In both the phagocytosis and the *in vivo* assay we also included a group where the coated apoptotic cells were pretreated with protein G to assess dependency on FcR-mediated regulation. We could indeed show that the increased response elicited by autoantibodies from serum from the memory response is at least in part FcR-dependent.

Paper II shows that there is a memory response towards the self-antigens present on apoptotic cells. It also shows that this memory response is more pathogenic than the initial break of tolerance and how the pathogenicity relates to the pathophysiology of SLE. It also sheds light on the fact that a lot but not all of the features in a classical memory response, which we have learned about from immune responses against foreign antigens, also hold true for this autoreactive memory. The findings in this study will be valuable for further studies on understanding how immune memory relates to SLE pathology and how to steer the response to self-antigens away from the part of the memory response leading to pathogenicity and worsened disease.

## 3.3 CD36 PLAYS A ROLE IN AUTOREACTIVE B CELL RESPONSES

CD36 is a scavenger receptor that is expressed on various immune cells but has mostly been studied on macrophages in the context of atherosclerosis for its ability to bind to epitopes on oxLDL [221]. CD36 is however also expressed on B cells and a few years ago it was found to be preferentially higher expressed on MZBs compared to other peripheral B cell subsets [51]. Being a scavenger receptor, CD36 binds to various ligands and amongst them antigens that express oxidation-specific epitopes, such as apoptotic cells. In paper III we investigated the role of CD36 on B cells in the context of autoimmune responses to modified self-antigens found on apoptotic cells and how this could affect subsequent B cell activation once tolerance to these antigens was broken.

To study these questions we used the same apoptotic cell model as described in paper I and II, which we applied or studied in a CD36-deficient (*CD36*$^{-/-}$) mouse. When characterizing the peripheral B cell compartment in the *CD36*$^{-/-}$ mouse we could not find any differences in the frequencies of T1 and T2 B cells or in the mature FOB and MZB subsets as compared to

wt controls. We also investigated how *CD36*$^{-/-}$ B cells responded to either the TI-II antigen NP-Ficoll or the TD antigen NP-CGG, using mixed bone marrow chimeras. The bone marrow chimeras were lethally irradiated wt mice that had received a mix of congenic wt and *CD36*$^{-/-}$ bone marrow or as controls wt mice with a mix of congenic wt and wt bone marrow. There were no major differences in the way B cells lacking CD36 responded to either the TI or TD antigen with regard to GC and plasma cell responses in the spleen. NP-specific antibody responses in sera of the *CD36*$^{-/-}$ bone marrow chimeras compared to controls were also measured and no difference in the response could be detected. CD36 therefore does not affect peripheral B cell development or the ability of B cells to respond to TI or TD antigens. However, we hypothesized that CD36 could be involved in the regulation of an immune response against self-antigens, such as those found on apoptotic cells. Mixed bone marrow chimeras, immunized with apoptotic cells, showed a significant expansion of *CD36*$^{-/-}$ B cells into GC B cells and unswitched plasma cells compared to wt B cells (Figure 7). *CD36*$^{-/-}$ chimeras compared to wt controls also exhibited increased levels of anti-DNA IgG antibodies. The significantly increased expansion of GC B cells and unswitched plasma cells in the *CD36*$^{-/-}$ B cell compartment indicated that CD36 plays an inhibitory role in the response to modified self-antigens and that this had consequences for the development of autoantibodies.

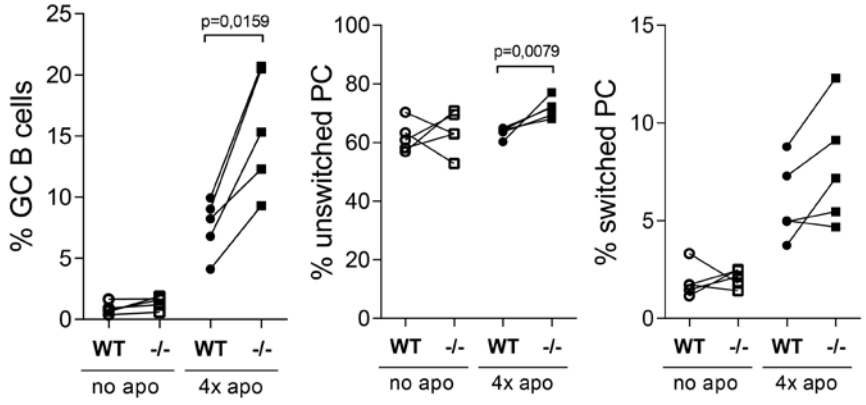


**Figure 7.** Frequency of GC B cells, unswitched and switched plasma cells (PC) in the spleen of mixed bone marrow chimeras with a mix of congenic wt and CD36 deficient (-/-) bone marrow. Mice were left untreated (no apo) or received apoptotic cell injections (4x apo). CD36 deficient B cells were more prone to enter the GC and differentiate to unswitched plasma cells, but here was no significant difference for switched plasma cells. n=5.

Next, we investigated how CD36 might convey the inhibitory effect observed in the response to apoptotic cells. Since CD36 does not have any signaling motifs of its own, it requires the engagement of a signaling partner. CD36 has been shown to associate with the tyrosine kinase Lyn in macrophages [208]. Performing a co-immunoprecipitation of Lyn and CD36 in a B cell line, we found that CD36 associated with Lyn also in B cells. Lyn has the ability to initiate activating signaling downstream of the BCR but it is also involved in inhibitory signaling, as it can phosphorylate the phosphatase SHIP downstream of FcγRIIb that in turn inhibits signaling through the BCR [96]. We therefore also investigated how CD36 interacted with the BCR and FcγRIIb using an advanced imaging technique where co-localization of receptors can be visualized and quantified on a single cell membrane [222]. At steady state, CD36 co-localized with the BCR, but upon crosslinking of FcγRIIb CD36 instead co-

localized with this receptor (Figure 8). Based on this data, associating CD36 with both Lyn and FcγRIIb, we next investigated SHIP phosphorylation in wt and *CD36*$^{-/-}$ B cells. Levels of phosphorylated SHIP were similar in anti-IgM stimulated wt and *CD36*$^{-/-}$ B cells, which led us to believe that other signaling proteins or pathways downstream of the BCR or FcγRIIb were involved. A possible candidate was the pathway regulated by Btk and JNK to induce apoptotic signaling as a consequence of IC crosslinking of FcγRIIb [99]. More evidence to support a role for CD36 in the IC-induced signaling pathway was found by performing an *in vitro* plasma cell killing assay, where B cells from wt or *CD36*$^{-/-}$ mice were first stimulated with LPS to induce plasma cell generation and then crosslinked with either an anti-FcγRIIb antibody or an isotype control. After antibody crosslinking the levels of apoptotic plasma cells were measured using flow cytometry. As expected there was an increased level of apoptotic plasma cells from wt mice but not for *CD36*$^{-/-}$ mice, indicating that regulation of plasma cell apoptosis is at least in part CD36-dependent.

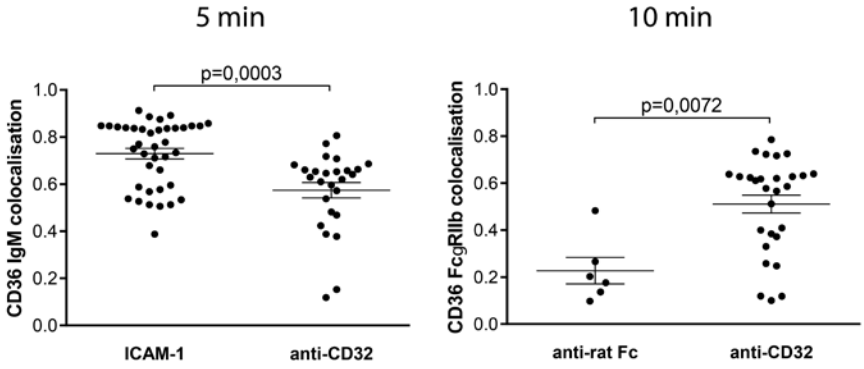


**Figure 8.** Pearson's co-localization coefficient for CD36 and IgM or FcγRIIB in images of CH27 cells at 5 and 10 min after being added to lipid bilayers containing either ICAM-1 alone, or ICAM-1 in combination with anti-rat Fc or anti-rat Fc + rat anti-CD32 antibody. The images were analyzed for co-localization and each symbol represents an individual cell. At steady state CD36 co-localized with the BCR but upon engagement of FcγRIIB, CD36 leaves the BCR and instead co-localizes with this receptor. n=6-39.

Another interesting observation in wt mice in the response to repeated apoptotic cell injections was that as the B cell response was induced over time, when self-tolerance was broken, the frequency of CD36-expressing MZBs was lowered. About two weeks after the last injection of apoptotic cells, the frequency of CD36$^{+}$ MZBs was restored to normal levels. The lowered CD36 expression could be due to down-regulation of CD36 on a transcriptional level, changes in the processes of internalization and recycling of the receptor or a preference for activation and proliferation of CD36-expressing MZBs into activated B cell subsets. Further studies are needed to answer these questions.

From the work using mouse models not a lot is known about the role of CD36 on B cells and even less is known about the expression pattern and role of CD36 on B cells in humans. We therefore sought to explore this, firstly by investigating the expression of CD36 on different peripheral B cell subsets using peripheral blood mononuclear cells (PBMC) from healthy donors. We found that CD36 was indeed expressed on naïve B cells, memory B cells as well as circulating MZBs. As opposed to CD36 expression in mouse it does not seem that MZBs, at least in the periphery, have a higher expression of CD36 compared to other B cell subsets. Having validated the expression of CD36, we moved on to compare levels of CD36-expressing B cell subsets in PBMCs from healthy donors compared to SLE patients. The

levels of CD36-expressing MZBs were significantly lower in the SLE patients compared to healthy individuals, although there was no major difference in levels of memory B cells or total B cells expressing CD36. This was a very interesting and potentially important finding as it correlated with the lowered CD36 expression we had observed in mice following apoptotic cell injections.

Paper III shows an inhibitory role for CD36 on B cells in the autoreactive immune response to modified self-antigens with consequences for the subsequent expansion of activated B cell subsets and autoantibody responses. CD36 most likely accomplishes this regulation through associating with known regulators of inhibitory B cell signaling, namely FcγRIIb and Lyn. It also shows a role for CD36 in regulating plasma cell apoptosis through an FcγRIIb-mediated pathway. This is an important means of regulation for the model we are studying, where ICs of autoantibodies and apoptotic cells are likely present and apoptosis induced by ICs is a mechanism to attenuate the immune response. It also shows how chronic systemic exposure to apoptotic cells decreases levels of MZBs expressing CD36. In SLE, where defects in removal of apoptotic cells are linked to risk for developing the disease, levels of peripheral MZB expressing CD36 are also lowered. Thus, finding ways to maintain CD36 expression on B cells could lead to new therapeutic targets.

## 3.4 FINAL REFLECTIONS AND FUTURE PERSPECTIVES

This thesis provides insight into how B cell responses are regulated in an autoimmune context and how this relates to the pathogenesis in atherosclerosis and SLE. More specifically the major findings are:

- The description of a protective B cell response in atherosclerosis originating in the spleen and induced by the oxidation-specific epitopes arising during sterile inflammation.
- An immunological memory response to apoptotic cell-derived self-antigens with some of the traits of a classic memory response and with implications for the pathogenesis of SLE.
- A role for the scavenger receptor CD36 on B cells in regulating an autoreactive immune response in a negative manner by associating with known B cell inhibitory components Lyn and FcγRIIB.

Paper II characterizes the memory response to apoptotic cell-derived self-antigens and shows how it relates to SLE pathology. Except for one published study where an increase in anti-cardiolipin antibodies upon re-immunization with apoptotic cells was observed, our study is first of its kind to show a memory response to apoptotic cell-derived self-antigens and how this memory response exhibits a lot of the same features as classic immunological memory, but interestingly also lack some, such as affinity maturation [143]. Disease flares in SLE patients are usually caused by environmental triggers, which can also lead to a rapid increase in the load of apoptotic cells. Hence, the finding that boosts of apoptotic cells following primary immunization led to memory activation accompanied by increased pathology for every subsequent recall response was an interesting link to flares in SLE patients. It is also an important finding as we could characterize this memory response and highlight what cellular components and specific antibody responses get reactivated and therefore could be involved

in the initiation of these flares. To further support the relevance of our findings for SLE pathology we found, using the autoantigen array, that the autoantibody profile in the memory response was selective to several different apoptotic cell-derived self-antigens. The strongest antibody response in the memory response was against the nuclear antigen Sm/RNP, which has not only been linked to SLE pathology in humans but is actually one of the serological diagnostic criterions. There was an increase in GC B cells and $T_{FH}$ cells in the memory response but at least for the anti-PC response there was no affinity maturation in the recall antibody response. However, since antibody responses towards several apoptotic cell-derived antigens were present in the memory response, it remains to be investigated whether antibodies with those reactivities have gone through affinity maturation. There was also a clear expansion of extrafollicular foci with both unswitched and isotype switched plasma cells. This leaves one wondering if the follicular or extrafollicular activation pathway is of more importance for an autoreactive memory response and to what degree the memory B cells require T cell help to get reactivated. To answer some of these questions an AID-reporter mouse could be used where all B cells that have expressed AID, and therefore would have received T cell help, will be GFP-tagged. It would demonstrate how abundant the contribution of T cell help is to the formation of the extrafollicular foci in the memory activation. It should however be mentioned that this system is complicated by the fact that also developing B cells can express AID to some extent in a TI manner with the use of BCR and TLR signaling alone [223]. Another approach could be to in more detail characterize the memory B cells in this model of autoimmune memory with specific memory markers such as CD80, CD73 and CD35. Apart from actually showing the presence of bonafide memory B cells in the model, these markers can also be correlated to V region mutation status [224].

Paper I dissects the protective B cell response in the spleen of atherosclerotic *Apoe*$^{-/-}$ mice, as transfer of B cells from atherosclerotic mice to young non-atherosclerotic *Apoe*$^{-/-}$ mice had previously been shown to protect against disease development [183]. The role of B cells in atherosclerosis is only beginning to be unraveled and when it comes to an atheroprotective role for B2 cells, published work shows contradictory results. Although some studies of anti-CD20 B2 cell depletion shows a positive correlation with disease outcome, a problem with these studies is that they do not discriminate between the contributions of FOBs versus MZBs. It is likely that the protective effect seen is mostly attributed to depletion of the FOBs, as these are more prone to engage in TD responses and can therefore possibly produce more pathogenic antibodies that could increase plaque development and instability. MZBs on the other hand, more readily engage in TI responses and are known producers of anti-PC IgM, both as natural antibodies and in response to *S. Pneumoniae* [225]. In paper I we found a striking expansion of the MZB pool but no increased expansion of FOBs in the old *Apoe*$^{-/-}$ mice, suggesting that the MZBs are a more likely candidate subset for conveying the protective effect. We cannot rule out the contribution of protective effects contributed by B1 cells in our study but B1 cells were not expanded in the aged mice either in the spleen or the peritoneum. The role of MZBs in atherosclerosis needs to be further elucidated and one approach could be to cross the *Apoe*$^{-/-}$ strain to a mouse strain with specific deletion of MZBs to assess the actual contribution to atheroprotection. To avoid defects associated with a lack of MZBs another approach could be to use an *Apoe*$^{-/-}$ mouse with MZBs with a mutated BCR lacking affinity for oxLDL. In paper I we were also able to induce the atheroprotective B cell response by immunizing young non-atherosclerotic *Apoe*$^{-/-}$ mice with apoptotic cells with

some of the same oxidation-specific epitopes as those present on modified lipids. It was very interesting to discover that this immunization was driving a B cell response very similar to the spontaneous ongoing B cell response in old atherosclerotic mice. This means that if the protective response can be induced at an early stage it can protect from disease development. The use of vaccination strategies for atherosclerosis is undeniably something that is emerging as a therapeutic approach and the body of work where different vaccination models have been tested in pre-clinical trials now sets the stage for large long-term clinical studies [226]. As a continuation of this project we aim to use the concept of autoimmune memory shown in paper II to induce a primary transient immune response against apoptotic cells in a mouse strain where the deletion of ApoE can be induced with tamoxifen treatment. This means we can control the onset of disease and we hypothesize that memory to apoptotic cell-derived self-antigens can be recalled by the induction of oxidation-specific epitopes arising during the course of atherosclerosis development and that the more rapid memory response could protect from plaque formation and disease. Something to keep in mind though is that the autoimmune memory response in paper II was accompanied by increased kidney pathology. However, the memory response could behave differently when it is evoked by atherosclerosis-associated antigens sharing molecular mimicry with apoptotic cells and could therefore result in a more protective phenotype. This remains to be evaluated.

Paper III demonstrates a role for the scavenger receptor CD36 on B cells in negatively regulating the autoimmune response to apoptotic cells. We have found that CD36 can associate with both Lyn and FcγRIIB, which are known negative regulators of B cell activation. It is therefore likely that CD36 upon binding apoptotic cell-derived self-antigens associates with these receptors to exert its inhibitory effects by affecting subsequent downstream signaling. That CD36 upon ligand binding can influence intracellular signaling has been shown for macrophages in numerous studies. The association with Lyn makes it possible for CD36 to activate SHIP downstream of FcγRIIB. However, *in vitro* stimulation assays of wt B cells compared to CD36-deficient B cells show no major difference in SHIP phosphorylation. We will therefore look further into a SHIP-independent pathway downstream of FcγRIIB involving Btk and JNK. This pathway is important for selection in the GC and in plasma cell apoptosis and is induced by ICs, which would likely be present in increased amounts following repeated injections of apoptotic cells. The *in vitro* data showing that plasma cell apoptosis following cross-linking of FcγRIIB is CD36-dependent further supports a role for CD36 in regulating this pathway. In addition CD36 has been shown to induce JNK-signaling in human cell lines [207]. Further studies involving *in vivo* and *in vitro* experiments with ICs with self-antigens are needed to discern the signaling pathways in B cells affected by CD36 in this model. After repeated apoptotic cell injections and break of tolerance to self we found that levels of CD36-expressing MZBs was dramatically decreased. Whether this is due to increased internalization, downregulation or an increased activation and differentiation of $CD36^+$ MZBs into GC B cells and plasma cells remains to be investigated. This however will be an important direction of the project since our investigations of CD36 expression on human peripheral B cells in SLE patients led us to find that similarly to the findings in wt mice, SLE patients have lower levels of $CD36^+$ peripheral circulating MZBs. To investigate how FcγRIIB expression correlates to CD36 expression on human B cells as well as to investigate *in vitro* signaling on CD36 expressing B cells in SLE patients compared to healthy individuals will be of great value. A few years back it was

established that CD36 expression in B cells is dependent on a different transcription factor than in macrophages or DCs, suggesting that a different transcriptional network could also be involved in the signaling and function of CD36 on B cells. Unraveling this network could provide more clues to the role of CD36 on B cells in different contexts.

I would like to conclude that the work in this thesis has led to the discovery of an inducible atheroprotective B cell response, characterization of the autoimmune memory response to apoptotic cell-derived self-antigens and the discovery of CD36 as a regulator of autoreactive B cell responses. The results provide a deeper understanding for the complexity of B cell regulation in autoimmunity and have implications for the treatment of atherosclerosis and SLE.

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# 5 REFERENCES


1. Murphy, K. and C. Weaver, *Janeway's Immunobiology*. 9 ed. 2016, New York: Garland Science.
2. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nat Immunol, 2015. **16**(4): p. 343-53.
3. Zouali, M. and Y. Richard, *Marginal zone B-cells, a gatekeeper of innate immunity.* Front Immunol, 2011. **2**: p. 63.
4. Medzhitov, R. and C.A. Janeway, Jr., *Innate immunity: impact on the adaptive immune response.* Curr Opin Immunol, 1997. **9**(1): p. 4-9.
5. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
6. Hoebe, K., et al., *CD36 is a sensor of diacylglycerides.* Nature, 2005. **433**(7025): p. 523-7.
7. Peiser, L., S. Mukhopadhyay, and S. Gordon, *Scavenger receptors in innate immunity.* Curr Opin Immunol, 2002. **14**(1): p. 123-8.
8. Moore, K.J. and M.W. Freeman, *Scavenger receptors in atherosclerosis: beyond lipid uptake.* Arterioscler Thromb Vasc Biol, 2006. **26**(8): p. 1702-11.
9. Jordo, E.D., et al., *Scavenger receptors as regulators of natural antibody responses and B cell activation in autoimmunity.* Mol Immunol, 2011. **48**(11): p. 1307-18.
10. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. **296**(5566): p. 301-5.
11. Chen, G.Y. and G. Nunez, *Sterile inflammation: sensing and reacting to damage.* Nat Rev Immunol, 2010. **10**(12): p. 826-37.
12. Nuki, G. and P.A. Simkin, *A concise history of gout and hyperuricemia and their treatment.* Arthritis Res Ther, 2006. **8 Suppl 1**: p. S1.
13. Miller, Y.I., et al., *Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity.* Circ Res, 2011. **108**(2): p. 235-48.
14. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta.* Mol Cell, 2002. **10**(2): p. 417-26.
15. Sims, J.E. and D.E. Smith, *The IL-1 family: regulators of immunity.* Nat Rev Immunol, 2010. **10**(2): p. 89-102.
16. Kumar, H., et al., *Involvement of the NLRP3 inflammasome in innate and humoral adaptive immune responses to fungal beta-glucan.* J Immunol, 2009. **183**(12): p. 8061-7.
17. Duewell, P., et al., *NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals.* Nature, 2010. **464**(7293): p. 1357-61.

18. Bendelac, A., M. Bonneville, and J.F. Kearney, *Autoreactivity by design: innate B and T lymphocytes.* Nat Rev Immunol, 2001. **1**(3): p. 177-86.

19. Cerutti, A., M. Cols, and I. Puga, *Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes.* Nat Rev Immunol, 2013. **13**(2): p. 118-32.

20. Lewis, S.M., *The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses.* Adv Immunol, 1994. **56**: p. 27-150.

21. Hozumi, N. and S. Tonegawa, *Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions.* Proc Natl Acad Sci U S A, 1976. **73**(10): p. 3628-32.

22. Brack, C., et al., *A complete immunoglobulin gene is created by somatic recombination.* Cell, 1978. **15**(1): p. 1-14.

23. Fugmann, S.D., et al., *The RAG proteins and V(D)J recombination: complexes, ends, and transposition.* Annu Rev Immunol, 2000. **18**: p. 495-527.

24. Oettinger, M.A., et al., *RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination.* Science, 1990. **248**(4962): p. 1517-23.

25. Bach, F.H., *Genetics of transplantation: the major histocompatibility complex.* Annu Rev Genet, 1976. **10**: p. 319-39.

26. Yamane, H. and W.E. Paul, *Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets.* Immunol Rev, 2013. **252**(1): p. 12-23.

27. Yu, D. and C.G. Vinuesa, *The elusive identity of T follicular helper cells.* Trends Immunol, 2010. **31**(10): p. 377-83.

28. Berken, A. and B. Benacerraf, *Properties of antibodies cytophilic for macrophages.* J Exp Med, 1966. **123**(1): p. 119-44.

29. Ravetch, J.V. and S. Bolland, *IgG Fc receptors.* Annu Rev Immunol, 2001. **19**: p. 275-90.

30. Takai, T., *Roles of Fc receptors in autoimmunity.* Nat Rev Immunol, 2002. **2**(8): p. 580-92.

31. Ahmed, R. and D. Gray, *Immunological memory and protective immunity: understanding their relation.* Science, 1996. **272**(5258): p. 54-60.

32. Sarkander, J., S. Hojyo, and K. Tokoyoda, *Vaccination to gain humoral immune memory.* Clin Transl Immunology, 2016. **5**(12): p. e120.

33. Slifka, M.K., et al., *Humoral immunity due to long-lived plasma cells.* Immunity, 1998. **8**(3): p. 363-72.

34. Radbruch, A., et al., *Competence and competition: the challenge of becoming a long-lived plasma cell.* Nat Rev Immunol, 2006. **6**(10): p. 741-50.

35. Kurosaki, T., K. Kometani, and W. Ise, *Memory B cells.* Nat Rev Immunol, 2015. **15**(3): p. 149-59.

36. Perdiguero, E.G. and F. Geissmann, *The development and maintenance of resident macrophages.* Nat Immunol, 2016. **17**(1): p. 2-8.

37. Baumgarth, N., *The double life of a B-1 cell: self-reactivity selects for protective effector functions.* Nat Rev Immunol, 2011. **11**(1): p. 34-46.

38. Mebius, R.E. and G. Kraal, *Structure and function of the spleen.* Nat Rev Immunol, 2005. **5**(8): p. 606-16.

39. Kraal, G. and R. Mebius, *New insights into the cell biology of the marginal zone of the spleen.* Int Rev Cytol, 2006. **250**: p. 175-215.

40. Gray, D., et al., *Relation of intra-splenic migration of marginal zone B cells to antigen localization on follicular dendritic cells.* Immunology, 1984. **52**(4): p. 659-69.

41. Cooper, M.D., R.D. Peterson, and R.A. Good, *Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken.* Nature, 1965. **205**: p. 143-6.

42. Wesemann, D.R., et al., *Microbial colonization influences early B-lineage development in the gut lamina propria.* Nature, 2013. **501**(7465): p. 112-5.

43. Wardemann, H., et al., *Predominant autoantibody production by early human B cell precursors.* Science, 2003. **301**(5638): p. 1374-7.

44. Eris, J.M., et al., *Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions.* Proc Natl Acad Sci U S A, 1994. **91**(10): p. 4392-6.

45. Goodnow, C.C., et al., *Induction of self-tolerance in mature peripheral B lymphocytes.* Nature, 1989. **342**(6248): p. 385-91.

46. Rice, J.S., et al., *Receptor editing in peripheral B cell tolerance.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1608-13.

47. Geisberger, R., M. Lamers, and G. Achatz, *The riddle of the dual expression of IgM and IgD.* Immunology, 2006. **118**(4): p. 429-37.

48. Allman, D. and S. Pillai, *Peripheral B cell subsets.* Curr Opin Immunol, 2008. **20**(2): p. 149-57.

49. Loder, F., et al., *B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals.* J Exp Med, 1999. **190**(1): p. 75-89.

50. Allman, D., et al., *Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation.* J Immunol, 2001. **167**(12): p. 6834-40.

51. Won, W.J., M.F. Bachmann, and J.F. Kearney, *CD36 is differentially expressed on B cell subsets during development and in responses to antigen.* J Immunol, 2008. **180**(1): p. 230-7.

52. Cinamon, G., et al., *Follicular shuttling of marginal zone B cells facilitates antigen transport.* Nat Immunol, 2008. **9**(1): p. 54-62.

53. Leadbetter, E.A., et al., *NK T cells provide lipid antigen-specific cognate help for B cells.* Proc Natl Acad Sci U S A, 2008. **105**(24): p. 8339-44.

54. Martin, F. and J.F. Kearney, *Marginal-zone B cells.* Nat Rev Immunol, 2002. **2**(5): p. 323-35.

55. Pillai, S. and A. Cariappa, *The follicular versus marginal zone B lymphocyte cell fate decision.* Nat Rev Immunol, 2009. **9**(11): p. 767-77.

56. Casola, S., et al., *B cell receptor signal strength determines B cell fate.* Nat Immunol, 2004. **5**(3): p. 317-27.

57. Herzenberg, L.A., *B-1 cells: the lineage question revisited.* Immunol Rev, 2000. **175**: p. 9-22.

58. Bos, N.A., et al., *Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet.* Eur J Immunol, 1989. **19**(12): p. 2335-9.

59. Rosser, E.C. and C. Mauri, *Regulatory B cells: origin, phenotype, and function.* Immunity, 2015. **42**(4): p. 607-12.

60. Evans, J.G., et al., *Novel suppressive function of transitional 2 B cells in experimental arthritis.* J Immunol, 2007. **178**(12): p. 7868-78.

61. Yanaba, K., et al., *A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses.* Immunity, 2008. **28**(5): p. 639-50.

62. Gray, M., et al., *Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells.* Proc Natl Acad Sci U S A, 2007. **104**(35): p. 14080-5.

63. Matsumoto, M., et al., *Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation.* Immunity, 2014. **41**(6): p. 1040-51.

64. Maseda, D., et al., *Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo.* J Immunol, 2012. **188**(3): p. 1036-48.

65. Barr, T.A., et al., *TLR-mediated stimulation of APC: Distinct cytokine responses of B cells and dendritic cells.* Eur J Immunol, 2007. **37**(11): p. 3040-53.

66. Czerkinsky, C.C., et al., *Reverse enzyme-linked immunospot assay (RELISPOT) for the detection of cells secreting immunoreactive substances.* J Immunol Methods, 1984. **72**(2): p. 489-96.

67. Weill, J.C., S. Weller, and C.A. Reynaud, *Human marginal zone B cells.* Annu Rev Immunol, 2009. **27**: p. 267-85.

68. Bemark, M., *Translating transitions - how to decipher peripheral human B cell development.* J Biomed Res, 2015. **29**(4): p. 264-84.

69. Cerutti, A., I. Puga, and M. Cols, *New helping friends for B cells.* Eur J Immunol, 2012. **42**(8): p. 1956-68.

70. Mongini, P.K., K.E. Stein, and W.E. Paul, *T cell regulation of IgG subclass antibody production in response to T-independent antigens.* J Exp Med, 1981. **153**(1): p. 1-12.

71. Kearney, J.F., *Innate-like B cells.* Springer Semin Immunopathol, 2005. **26**(4): p. 377-83.

72. Vinuesa, C.G. and P.P. Chang, *Innate B cell helpers reveal novel types of antibody responses.* Nat Immunol, 2013. **14**(2): p. 119-26.

73. de Vinuesa, C.G., et al., *Germinal centers without T cells.* J Exp Med, 2000. **191**(3): p. 485-94.

74. Obukhanych, T.V. and M.C. Nussenzweig, *T-independent type II immune responses generate memory B cells.* J Exp Med, 2006. **203**(2): p. 305-10.

75. Tas, J.M., et al., *Visualizing antibody affinity maturation in germinal centers.* Science, 2016. **351**(6277): p. 1048-54.

76. Muramatsu, M., et al., *Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells.* J Biol Chem, 1999. **274**(26): p. 18470-6.

77. Aguzzi, A., J. Kranich, and N.J. Krautler, *Follicular dendritic cells: origin, phenotype, and function in health and disease.* Trends Immunol, 2014. **35**(3): p. 105-13.

78. Smith, J.P., et al., *Tingible body macrophages in regulation of germinal center reactions.* Dev Immunol, 1998. **6**(3-4): p. 285-94.

79. Vinuesa, C.G., I. Sanz, and M.C. Cook, *Dysregulation of germinal centres in autoimmune disease.* Nat Rev Immunol, 2009. **9**(12): p. 845-57.

80. Castigli, E., et al., *TACI and BAFF-R mediate isotype switching in B cells.* J Exp Med, 2005. **201**(1): p. 35-9.

81. Shulman, Z., et al., *Dynamic signaling by T follicular helper cells during germinal center B cell selection.* Science, 2014. **345**(6200): p. 1058-62.

82. Pelletier, N., et al., *The endoplasmic reticulum is a key component of the plasma cell death pathway.* J Immunol, 2006. **176**(3): p. 1340-7.

83. Shaffer, A.L., et al., *Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program.* Immunity, 2002. **17**(1): p. 51-62.

84. MacLennan, I.C., et al., *Extrafollicular antibody responses.* Immunol Rev, 2003. **194**: p. 8-18.

85. Sze, D.M., et al., *Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival.* J Exp Med, 2000. **192**(6): p. 813-21.

86. Hargreaves, D.C., et al., *A coordinated change in chemokine responsiveness guides plasma cell movements.* J Exp Med, 2001. **194**(1): p. 45-56.

87. Garcia De Vinuesa, C., et al., *Dendritic cells associated with plasmablast survival.* Eur J Immunol, 1999. **29**(11): p. 3712-21.

88. Taylor, J.J., K.A. Pape, and M.K. Jenkins, *A germinal center-independent pathway generates unswitched memory B cells early in the primary response.* J Exp Med, 2012. **209**(3): p. 597-606.

89. Klein, U., R. Kuppers, and K. Rajewsky, *Evidence for a large compartment of IgM-expressing memory B cells in humans.* Blood, 1997. **89**(4): p. 1288-98.

90. Schwickert, T.A., et al., *A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center.* J Exp Med, 2011. **208**(6): p. 1243-52.

91. Qi, H., et al., *SAP-controlled T-B cell interactions underlie germinal centre formation.* Nature, 2008. **455**(7214): p. 764-9.

92. Linterman, M.A., et al., *IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses.* J Exp Med, 2010. **207**(2): p. 353-63.

93. Weisel, F.J., et al., *A Temporal Switch in the Germinal Center Determines Differential Output of Memory B and Plasma Cells.* Immunity, 2016. **44**(1): p. 116-30.

94. Nutt, S.L., et al., *The generation of antibody-secreting plasma cells.* Nat Rev Immunol, 2015. **15**(3): p. 160-71.

95. Alugupalli, K.R., et al., *B1b lymphocytes confer T cell-independent long-lasting immunity.* Immunity, 2004. **21**(3): p. 379-90.

96. Niiro, H. and E.A. Clark, *Regulation of B-cell fate by antigen-receptor signals.* Nat Rev Immunol, 2002. **2**(12): p. 945-56.

97. Scharenberg, A.M., L.A. Humphries, and D.J. Rawlings, *Calcium signalling and cell-fate choice in B cells.* Nat Rev Immunol, 2007. **7**(10): p. 778-89.

98. Srinivasan, L., et al., *PI3 kinase signals BCR-dependent mature B cell survival.* Cell, 2009. **139**(3): p. 573-86.

99. Pritchard, N.R. and K.G. Smith, *B cell inhibitory receptors and autoimmunity.* Immunology, 2003. **108**(3): p. 263-73.

100. Limon, J.J. and D.A. Fruman, *Akt and mTOR in B Cell Activation and Differentiation.* Front Immunol, 2012. **3**: p. 228.

101. Pearse, R.N., et al., *SHIP recruitment attenuates Fc gamma RIIB-induced B cell apoptosis.* Immunity, 1999. **10**(6): p. 753-60.

102. Tzeng, S.J., et al., *The B cell inhibitory Fc receptor triggers apoptosis by a novel c-Abl family kinase-dependent pathway.* J Biol Chem, 2005. **280**(42): p. 35247-54.

103. Bolland, S. and J.V. Ravetch, *Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis.* Immunity, 2000. **13**(2): p. 277-85.

104. Silverstein, A.M., *Autoimmunity versus horror autotoxicus: the struggle for recognition.* Nat Immunol, 2001. **2**(4): p. 279-81.

105. Chan, O.T., M.P. Madaio, and M.J. Shlomchik, *The central and multiple roles of B cells in lupus pathogenesis.* Immunol Rev, 1999. **169**: p. 107-21.

106. Edwards, J.C., et al., *Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis.* N Engl J Med, 2004. **350**(25): p. 2572-81.

107. Serreze, D.V., et al., *B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice.* J Exp Med, 1996. **184**(5): p. 2049-53.

108. Antel, J. and A. Bar-Or, *Roles of immunoglobulins and B cells in multiple sclerosis: from pathogenesis to treatment.* J Neuroimmunol, 2006. **180**(1-2): p. 3-8.

109. Liossis, S.N. and P.P. Sfikakis, *Rituximab-induced B cell depletion in autoimmune diseases: potential effects on T cells.* Clin Immunol, 2008. **127**(3): p. 280-5.

110. Cyster, J.G. and C.C. Goodnow, *Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate.* Immunity, 1995. **3**(6): p. 691-701.

111. Mackay, F., et al., *Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations.* J Exp Med, 1999. **190**(11): p. 1697-710.

112. Groom, J., et al., *Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome.* J Clin Invest, 2002. **109**(1): p. 59-68.

113. Thien, M., et al., *Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches.* Immunity, 2004. **20**(6): p. 785-98.

114. Jacobson, B.A., et al., *Anatomy of autoantibody production: dominant localization of antibody-producing cells to T cell zones in Fas-deficient mice.* Immunity, 1995. **3**(4): p. 509-19.

115. William, J., et al., *Evolution of autoantibody responses via somatic hypermutation outside of germinal centers.* Science, 2002. **297**(5589): p. 2066-70.

116. Hoyer, B.F., et al., *Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice.* J Exp Med, 2004. **199**(11): p. 1577-84.

117. Leadbetter, E.A., et al., *Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors.* Nature, 2002. **416**(6881): p. 603-7.

118. Lau, C.M., et al., *RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement.* J Exp Med, 2005. **202**(9): p. 1171-7.

119. Jegerlehner, A., et al., *TLR9 signaling in B cells determines class switch recombination to IgG2a.* J Immunol, 2007. **178**(4): p. 2415-20.

120. William, J., C. Euler, and M.J. Shlomchik, *Short-lived plasmablasts dominate the early spontaneous rheumatoid factor response: differentiation pathways, hypermutating cell types, and affinity maturation outside the germinal center.* J Immunol, 2005. **174**(11): p. 6879-87.

121. Qi, H., et al., *Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells.* Science, 2006. **312**(5780): p. 1672-6.

122. Treml, J.F., et al., *The BLyS family: toward a molecular understanding of B cell homeostasis.* Cell Biochem Biophys, 2009. **53**(1): p. 1-16.

123. Carter, N.A., E.C. Rosser, and C. Mauri, *Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis.* Arthritis Res Ther, 2012. **14**(1): p. R32.

124. Kinnunen, T., et al., *Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells.* Blood, 2013. **121**(9): p. 1595-603.

125. Carter, N.A., et al., *Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells.* J Immunol, 2011. **186**(10): p. 5569-79.

126. Paul, E., et al., *Follicular exclusion of autoreactive B cells requires FcgammaRIIb.* Int Immunol, 2007. **19**(4): p. 365-73.

127. Avalos, A.M., et al., *FcgammaRIIB regulation of BCR/TLR-dependent autoreactive B-cell responses.* Eur J Immunol, 2010. **40**(10): p. 2692-8.

128. Nishizumi, H., et al., *Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice.* Immunity, 1995. **3**(5): p. 549-60.

129. Hibbs, M.L., et al., *Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease.* Cell, 1995. **83**(2): p. 301-11.

130. Lamagna, C., et al., *B cell-specific loss of Lyn kinase leads to autoimmunity.* J Immunol, 2014. **192**(3): p. 919-28.

131. Danchenko, N., J.A. Satia, and M.S. Anthony, *Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden.* Lupus, 2006. **15**(5): p. 308-18.

132. Buyon, J.P., et al., *The effect of combined estrogen and progesterone hormone replacement therapy on disease activity in systemic lupus erythematosus: a randomized trial.* Ann Intern Med, 2005. **142**(12 Pt 1): p. 953-62.

133. Cohen-Solal, J.F., et al., *Sex hormones and SLE: influencing the fate of autoreactive B cells.* Curr Top Microbiol Immunol, 2006. **305**: p. 67-88.

134. Hochberg, M.C., *Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus.* Arthritis Rheum, 1997. **40**(9): p. 1725.

135. Sullivan, K.E., *Genetics of systemic lupus erythematosus. Clinical implications.* Rheum Dis Clin North Am, 2000. **26**(2): p. 229-56, v-vi.

136. Kramers, C., et al., *Anti-nucleosome antibodies complexed to nucleosomal antigens show anti-DNA reactivity and bind to rat glomerular basement membrane in vivo.* J Clin Invest, 1994. **94**(2): p. 568-77.

137. Gaipl, U.S., et al., *Clearance of apoptotic cells in human SLE.* Curr Dir Autoimmun, 2006. **9**: p. 173-87.

138. Ren, Y., et al., *Increased apoptotic neutrophils and macrophages and impaired macrophage phagocytic clearance of apoptotic neutrophils in systemic lupus erythematosus.* Arthritis Rheum, 2003. **48**(10): p. 2888-97.

139. Botto, M., et al., *Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies.* Nat Genet, 1998. **19**(1): p. 56-9.

140. Scott, R.S., et al., *Phagocytosis and clearance of apoptotic cells is mediated by MER.* Nature, 2001. **411**(6834): p. 207-11.

141. Hanayama, R., et al., *Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice.* Science, 2004. **304**(5674): p. 1147-50.

142. Li, M.O., et al., *Phosphatidylserine receptor is required for clearance of apoptotic cells.* Science, 2003. **302**(5650): p. 1560-3.

143. Mevorach, D., et al., *Systemic exposure to irradiated apoptotic cells induces autoantibody production.* J Exp Med, 1998. **188**(2): p. 387-92.

144. Mukundan, L., et al., *PPAR-delta senses and orchestrates clearance of apoptotic cells to promote tolerance.* Nat Med, 2009. **15**(11): p. 1266-72.

145. Enzler, T., et al., *Alternative and classical NF-kappa B signaling retain autoreactive B cells in the splenic marginal zone and result in lupus-like disease.* Immunity, 2006. **25**(3): p. 403-15.

146. Grader-Beck, T., et al., *Apoptotic splenocytes drive the autoimmune response to poly(ADP-ribose) polymerase 1 in a murine model of lupus.* J Immunol, 2007. **178**(1): p. 95-102.

147. O'Brien, B.A., et al., *A deficiency in the in vivo clearance of apoptotic cells is a feature of the NOD mouse.* J Autoimmun, 2006. **26**(2): p. 104-15.

148. Wermeling, F., et al., *Class A scavenger receptors regulate tolerance against apoptotic cells, and autoantibodies against these receptors are predictive of systemic lupus.* J Exp Med, 2007. **204**(10): p. 2259-65.

149. Henson, P.M. and D.L. Bratton, *Antiinflammatory effects of apoptotic cells.* J Clin Invest, 2013. **123**(7): p. 2773-4.

150. Rock, K.L. and H. Kono, *The inflammatory response to cell death.* Annu Rev Pathol, 2008. **3**: p. 99-126.

151. Roman, M.J., et al., *Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus.* N Engl J Med, 2003. **349**(25): p. 2399-406.

152. Murray, C.J. and A.D. Lopez, *Measuring the global burden of disease.* N Engl J Med, 2013. **369**(5): p. 448-57.

153. Bentzon, J.F., et al., *Mechanisms of plaque formation and rupture.* Circ Res, 2014. **114**(12): p. 1852-66.

154. Ross, R., *Atherosclerosis--an inflammatory disease.* N Engl J Med, 1999. **340**(2): p. 115-26.

155. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.

156. Skeoch, S. and I.N. Bruce, *Atherosclerosis in rheumatoid arthritis: is it all about inflammation?* Nat Rev Rheumatol, 2015. **11**(7): p. 390-400.

157. Spah, F., *Inflammation in atherosclerosis and psoriasis: common pathogenic mechanisms and the potential for an integrated treatment approach.* Br J Dermatol, 2008. **159 Suppl 2**: p. 10-7.

158. Mestas, J. and K. Ley, *Monocyte-endothelial cell interactions in the development of atherosclerosis.* Trends Cardiovasc Med, 2008. **18**(6): p. 228-32.

159. Steinberg, D., *The LDL modification hypothesis of atherogenesis: an update.* J Lipid Res, 2009. **50 Suppl**: p. S376-81.

160. West, X.Z., et al., *Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands.* Nature, 2010. **467**(7318): p. 972-6.

161. Miller, Y.I., et al., *Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells.* J Biol Chem, 2003. **278**(3): p. 1561-8.

162. Bjorkbacka, H., et al., *Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways.* Nat Med, 2004. **10**(4): p. 416-21.

163. Tan, H.W., et al., *IL-18 overexpression promotes vascular inflammation and remodeling in a rat model of metabolic syndrome.* Atherosclerosis, 2010. **208**(2): p. 350-7.

164. de Nooijer, R., et al., *Overexpression of IL-18 decreases intimal collagen content and promotes a vulnerable plaque phenotype in apolipoprotein-E-deficient mice.* Arterioscler Thromb Vasc Biol, 2004. **24**(12): p. 2313-9.

165. Elhage, R., et al., *Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice.* Cardiovasc Res, 2003. **59**(1): p. 234-40.

166. Kirii, H., et al., *Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-deficient mice.* Arterioscler Thromb Vasc Biol, 2003. **23**(4): p. 656-60.

167. Hansson, G.K., J. Holm, and L. Jonasson, *Detection of activated T lymphocytes in the human atherosclerotic plaque.* Am J Pathol, 1989. **135**(1): p. 169-75.

168. Zhou, X., et al., *Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice.* Circulation, 2000. **102**(24): p. 2919-22.

169. Tedgui, A. and Z. Mallat, *Cytokines in atherosclerosis: pathogenic and regulatory pathways.* Physiol Rev, 2006. **86**(2): p. 515-81.

170. Gupta, S., et al., *IFN-gamma potentiates atherosclerosis in ApoE knock-out mice.* J Clin Invest, 1997. **99**(11): p. 2752-61.

171. Whitman, S.C., et al., *Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice.* Am J Pathol, 2000. **157**(6): p. 1819-24.

172. Klingenberg, R., et al., *Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis.* J Clin Invest, 2013. **123**(3): p. 1323-34.

173. Mallat, Z., et al., *Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice.* Circ Res, 2001. **89**(10): p. 930-4.

174. Caligiuri, G., et al., *Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice.* Mol Med, 2003. **9**(1-2): p. 10-7.

175. Tsiantoulas, D., et al., *B cells and humoral immunity in atherosclerosis.* Circ Res, 2014. **114**(11): p. 1743-56.

176. Galkina, E., et al., *Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent.* J Exp Med, 2006. **203**(5): p. 1273-82.

177. Binder, C.J., et al., *Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL.* Nat Med, 2003. **9**(6): p. 736-43.

178. Binder, C.J., et al., *IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis.* J Clin Invest, 2004. **114**(3): p. 427-37.

179. Caligiuri, G., et al., *Phosphorylcholine-targeting immunization reduces atherosclerosis.* J Am Coll Cardiol, 2007. **50**(6): p. 540-6.

180. Faria-Neto, J.R., et al., *Passive immunization with monoclonal IgM antibodies against phosphorylcholine reduces accelerated vein graft atherosclerosis in apolipoprotein E-null mice.* Atherosclerosis, 2006. **189**(1): p. 83-90.

181. Yla-Herttuala, S., et al., *Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL.* Arterioscler Thromb, 1994. **14**(1): p. 32-40.

182. Lichtman, A.H., et al., *Adaptive immunity in atherogenesis: new insights and therapeutic approaches.* J Clin Invest, 2013. **123**(1): p. 27-36.

183. Caligiuri, G., et al., *Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice.* J Clin Invest, 2002. **109**(6): p. 745-53.

184. Robinette, C.D. and J.F. Fraumeni, Jr., *Splenectomy and subsequent mortality in veterans of the 1939-45 war.* Lancet, 1977. **2**(8029): p. 127-9.

185. Major, A.S., S. Fazio, and M.F. Linton, *B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice.* Arterioscler Thromb Vasc Biol, 2002. **22**(11): p. 1892-8.

186. Ait-Oufella, H., et al., *B cell depletion reduces the development of atherosclerosis in mice.* J Exp Med, 2010. **207**(8): p. 1579-87.

187. Kyaw, T., et al., *Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis.* J Immunol, 2010. **185**(7): p. 4410-9.

188. Kyaw, T., et al., *Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation.* PLoS One, 2012. **7**(1): p. e29371.

189. Kyaw, T., et al., *BAFF receptor mAb treatment ameliorates development and progression of atherosclerosis in hyperlipidemic ApoE(-/-) mice.* PLoS One, 2013. **8**(4): p. e60430.

190. Doran, A.C., et al., *B-cell aortic homing and atheroprotection depend on Id3.* Circ Res, 2012. **110**(1): p. e1-12.

191. Kyaw, T., et al., *B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions.* Circ Res, 2011. **109**(8): p. 830-40.

192. Wardemann, H., et al., *B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen.* J Exp Med, 2002. **195**(6): p. 771-80.

193. Sage, A.P., et al., *Regulatory B cell-specific interleukin-10 is dispensable for atherosclerosis development in mice.* Arterioscler Thromb Vasc Biol, 2015. **35**(8): p. 1770-3.

194. Strom, A.C., et al., *B regulatory cells are increased in hypercholesterolaemic mice and protect from lesion development via IL-10.* Thromb Haemost, 2015. **114**(4): p. 835-47.

195. Febbraio, M., D.P. Hajjar, and R.L. Silverstein, *CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism.* J Clin Invest, 2001. **108**(6): p. 785-91.

196. Hart, K. and M. Wilcox, *A Drosophila gene encoding an epithelial membrane protein with homology to CD36/LIMP II.* J Mol Biol, 1993. **234**(1): p. 249-53.

197. Means, T.K., et al., *Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36.* J Exp Med, 2009. **206**(3): p. 637-53.

198. Oquendo, P., et al., *CD36 directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes.* Cell, 1989. **58**(1): p. 95-101.

199. Albert, M.L., et al., *Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes.* J Exp Med, 1998. **188**(7): p. 1359-68.

200. Endemann, G., et al., *CD36 is a receptor for oxidized low density lipoprotein.* J Biol Chem, 1993. **268**(16): p. 11811-6.

201. Podrez, E.A., et al., *Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species.* J Clin Invest, 2000. **105**(8): p. 1095-108.

202. Rahaman, S.O., et al., *A CD36-dependent signaling cascade is necessary for macrophage foam cell formation.* Cell Metab, 2006. **4**(3): p. 211-21.

203. Jimenez, B., et al., *Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1.* Nat Med, 2000. **6**(1): p. 41-8.

204. Silverstein, R.L. and M. Febbraio, *CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior.* Sci Signal, 2009. **2**(72): p. re3.

205. Heit, B., et al., *Multimolecular signaling complexes enable Syk-mediated signaling of CD36 internalization.* Dev Cell, 2013. **24**(4): p. 372-83.

206. Triantafilou, M., et al., *Lipopolysaccharides from atherosclerosis-associated bacteria antagonize TLR4, induce formation of TLR2/1/CD36 complexes in lipid rafts and trigger TLR2-induced inflammatory responses in human vascular endothelial cells.* Cell Microbiol, 2007. **9**(8): p. 2030-9.

207. Baranova, I.N., et al., *Role of human CD36 in bacterial recognition, phagocytosis, and pathogen-induced JNK-mediated signaling.* J Immunol, 2008. **181**(10): p. 7147-56.

208. Stewart, C.R., et al., *CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer.* Nat Immunol, 2010. **11**(2): p. 155-61.

209. Corcoran, L., et al., *Differential regulation of CD36 expression in antigen-presenting cells: Oct-2 dependence in B lymphocytes but not dendritic cells or macrophages.* Int Immunol, 2002. **14**(10): p. 1099-104.

210. Rutella, S., et al., *Expression of thrombospondin receptor (CD36) in B-cell chronic lymphocytic leukemia as an indicator of tumor cell dissemination.* Haematologica, 1999. **84**(5): p. 419-24.

211. Johnson, S.A., S.J. Rozzo, and J.C. Cambier, *Aging-dependent exclusion of antigen-inexperienced cells from the peripheral B cell repertoire.* J Immunol, 2002. **168**(10): p. 5014-23.

212. Ben-Sasson, S.Z., et al., *IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation.* Proc Natl Acad Sci U S A, 2009. **106**(17): p. 7119-24.

213. Rahman, A. and D.A. Isenberg, *Systemic lupus erythematosus.* N Engl J Med, 2008. **358**(9): p. 929-39.

214. Carroll, M.C., *The complement system in regulation of adaptive immunity.* Nat Immunol, 2004. **5**(10): p. 981-6.

215. Tangye, S.G., et al., *The good, the bad and the ugly - TFH cells in human health and disease.* Nat Rev Immunol, 2013. **13**(6): p. 412-26.

216. Kohler, H., *The response to phosphorylcholine: dissecting an immune response.* Transplant Rev, 1975. **27**: p. 24-56.

217. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.

218. Li, Q.Z., et al., *Identification of autoantibody clusters that best predict lupus disease activity using glomerular proteome arrays.* J Clin Invest, 2005. **115**(12): p. 3428-39.

219. Li, Q.Z., et al., *Protein array autoantibody profiles for insights into systemic lupus erythematosus and incomplete lupus syndromes.* Clin Exp Immunol, 2007. **147**(1): p. 60-70.

220. Migliorini, P., et al., *Anti-Sm and anti-RNP antibodies.* Autoimmunity, 2005. **38**(1): p. 47-54.

221. Collot-Teixeira, S., et al., *CD36 and macrophages in atherosclerosis.* Cardiovasc Res, 2007. **75**(3): p. 468-77.

222. Sohn, H.W., et al., *A method for analyzing protein-protein interactions in the plasma membrane of live B cells by fluorescence resonance energy transfer imaging as acquired by total internal reflection fluorescence microscopy.* Methods Mol Biol, 2010. **591**: p. 159-83.

223. Han, J.H., et al., *Class switch recombination and somatic hypermutation in early mouse B cells are mediated by B cell and Toll-like receptors.* Immunity, 2007. **27**(1): p. 64-75.

224. Anderson, S.M., et al., *New markers for murine memory B cells that define mutated and unmutated subsets.* J Exp Med, 2007. **204**(9): p. 2103-14.

225. Martin, F., A.M. Oliver, and J.F. Kearney, *Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens.* Immunity, 2001. **14**(5): p. 617-29.

226. Garcia-Gonzalez, V., et al., *Reality of a Vaccine in the Prevention and Treatment of Atherosclerosis.* Arch Med Res, 2015. **46**(5): p. 427-37.